PHARMACOKINETICS AND PHARMACODYNAMICS OF CEFACLOR

By

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Dedicated to my parents, my sisters and my husband.

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Abstract of Dissertation Presented to the Graduate School
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PHARMACOKINETICS AND PHARMACODYNAMICS OF CEFACLOR

By

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The aim of this study was to use microdialysis and bacterial kill curves to develop a pharmacokinetic-pharmacodynamic (PK-PD) model to describe the anti-infective effect of clinically active concentrations of antibiotics, at their site of action. A β-lactam cephalosporin antibiotic, cefaclor, was used for the study.

Pharmacokinetics: the plasma and free tissue pharmacokinetics of cefaclor in both muscle and lung were determined in rats after IV administration of 50 mg/Kg and 75 mg/Kg doses. Plasma and free thigh muscle pharmacokinetics of cefaclor were investigated in humans after oral administration. Two oral formulations, an immediate- (500 mg IR) and a modified-release formulation in two different doses (500 mg MR and 750 mg MR), were investigated in humans. Microdialysis was used to obtain the free tissue concentrations in both species. All samples were assayed by HPLC with UV detection at λ=254 nm. Protein binding was determined in both rat and human plasma. Using appropriate pharmacokinetic models for both species and formulations, free tissue levels could be predicted applying pharmacokinetic parameters derived from the plasma concentrations.

Pharmacodynamics: four microorganisms (^{a}E.coli ATCC®25922, ^{b}M.catarrhalis ATCC®25240, ^{c}H. influenzae ATCC®10211 and ^{d}S.pneumoniae ATCC®49619) were exposed to simulated human free tissue pharmacokinetics in an in vitro model of infection. The pharmacodynamic data were fitted with a modified E_{max} model.

The effect of different dosing regimens could be predicted using the PK-PD model. Anti-infective effectiveness was measured as the number of bacteria remaining after 24 hours (N_{24h}). Dosing regimens were compared using the ratio between N_{24h}.

The developed PK-PD model enables us to make rational decisions with respect to antibiotic dosing by easily predicting the effect of various dosing regimens, taking into account the clinically effective free concentrations at the site of action. Different types of drug administration (i.e., extended release formulations) could also be built into the model and evaluated. The results suggest there is an advantage to using the sustained release formulations versus the immediate release formulation.

CHAPTER 1
INTRODUCTION

Relevance of the Study

The present thesis applies the concept of pharmacokinetic/pharmacodynamic (PK/PD) modeling to the evaluation of a beta-lactam antibiotic. The treatment of bacterial infections is currently based more on clinical experience than on a rational scientific approach. To be able to develop such an approach and predict the outcome of the treatment of infections, the characteristics of the bacteria, the host and the drug need to be taken into account. Antimicrobial susceptibility and particularly resistance are rapidly becoming one of the major public concerns about modern medicine [1]. New treatment strategies will need to be devised in order for therapies to be effective. There are several approaches currently used to estimate the efficacy of antibiotics, all based on the minimum inhibitory concentration (MIC) of the anti-infective agents against the bacteria of interest [2]. Despite these efforts, for many antibiotics the results are still not satisfactory.

The use of an adequate dose and dosing interval may also have an impact on patient compliance and cost, as well as reducing the emergence of resistance. To reach that goal, this work investigates if less frequent administration of a modified formulation of a current antibiotic may have the same efficacy as frequent and rather tedious dosing schedules. An important step in addressing this question is the combination of pharmacokinetic parameters of the antibiotic (PK) with its pharmacodynamic properties against bacteria (PD) in a PK-PD model. Both parts together (PK and PD) can be used to predict the effect as a function of time and allow for a systematic study of interactions between drug and microorganism [3].

The information gathered in this work will allow us to rationally compare different doses and dosing regimens by using a PK-PD model and make accurate predictions based on a scientific approach.

Objectives

The objectives of the present study were:

1. to investigate the pharmacokinetics of the cephalosporin cefaclor in rats by analyzing plasma and tissue data from two different tissues,
2. to investigate the relationship between the concentration-time profiles in both lung and muscle tissues,
3. to integrate the plasma and tissue data in a pharmacokinetic model that would allow us to predict and simulate concentration-time profiles of cefaclor in rat tissues based on plasma levels,
4. to investigate the pharmacokinetics of cefaclor in humans by analyzing plasma and muscle tissue data after administration of two different cefaclor formulations in two different doses,
5. to compare the pharmacokinetics of the different cefaclor formulations,
6. to integrate the human plasma and tissue data in a pharmacokinetic model that would allow us to predict and simulate concentration-time profiles of cefaclor in human tissues based on plasma levels,

7. to evaluate the pharmacodynamic effect of cefaclor (changes in the number of bacteria as a function of time) in an *in vitro* model that allows for simulation of human tissue concentration profiles, against four different bacterial strains,
8. to describe the pharmacodynamic effect with an appropriate mathematical model that would allow to simulate the pharmacodynamic effect for any given dose and dosing regimen on any of the studied bacterial strains,
9. to find a simple way of comparing the different dosing regimens evaluated and make a recommendation for the optimal dosing regimen.

CHAPTER 2
LITERATURE REVIEW

PK/PD models

Antimicrobial susceptibility and particularly resistance are rapidly becoming one of the major public concerns in modern medicine. We have recently moved into an era of not just multiple resistant bacteria but of totally resistant pathogens [1]. In order for therapies to be effective, new treatment strategies will need to be devised.

Pharmacokinetic/pharmacodynamic (PK/PD) modeling recently has become a very vital field in drug development. Pharmacokinetics describes the fate of the drug in the body in terms of absorption, distribution, metabolism and elimination, using concentration-time profiles. Pharmacodynamics evaluates the effect of the drug against time, and PK/PD links the concentrations to the effect. The main concept is to utilize systematically the concentration-effect relationship of the drug of interest in dose finding and product development in a logical way and minimize trial-and-error approaches [4, 5]. It could be shown that this approach can result in major saving of time and expenses and avoid unnecessary and, hence, unethical clinical studies [6]. There are several types of PK/PD approaches described in the literature that can be adapted to particular situations.

The dosages and dosing intervals of antimicrobial agents should be designed with reference to PK and PD parameters. Accordingly, several efficacy indices or surrogate markers that take into account both PK and PD information have been defined and used by different authors to describe the antibacterial activity of various classes of antimicrobial agents [7-9].

There are currently two main trends for antibiotic PK/PD models; those based on the MIC and those based on a kill-curve approach.

Traditional Non-compartmental PK/PD Models Based on MIC

The most common approach to antibiotic dosing is to adjust the doses to obtain antibiotic plasma concentrations that are above the MIC for the respective pathogen throughout the dosing interval. The MIC is the lowest concentration that completely inhibits visible growth of the organism as detected by the unaided eye after a 18- to 24-hour incubation period with a standard inoculum of approximately 10^5 colony forming units [10].

In these approaches, the PK parameter is usually the serum concentration of the anti-infective agent, and the PD parameter is almost exclusively the minimum inhibitory concentration (MIC) [11-13]. The minimal bactericidal concentration (MBC), which is the lowest concentration of the antibiotic that causes complete destruction of the pathogen, has also been used for the same purpose [14-19].

Figure 1 shows the most frequently used parameters, which are: Time>MIC, Cmax/MIC, AUC/MIC as well as the area under the inhibitory curve AUIC that is calculated from the (AUC_t>MIC/MIC) over 24 hours [20].

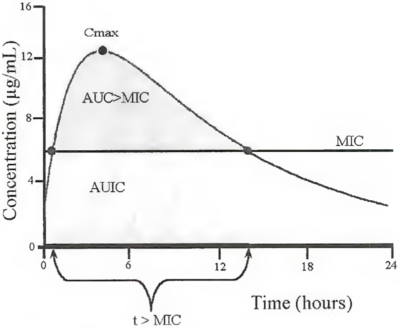


Figure 1: Currently used PK/PD parameters.

Antibiotics are usually divided into two major groups: those that exhibit time-dependent (concentration-independent) killing and minimally-to-moderately persistent effects and those that exhibit concentration-dependent killing and prolonged persistent effects [21].

For antibiotics that belong to the first group (beta-lactam antibiotics except carbapenems; clindamycin, macrolides), their effect depends on the length of time that the drug is in contact with the bacteria. At the beginning, the effect will increase with increasing concentrations until a finite point (the maximum kill rate) is reached. After that point, increasing concentrations will not produce a corresponding increase in the effect; therefore, high concentration peaks will not help. Zero-order killing has been seen to occur at concentrations approximately four to five times the MIC [22]. A paradoxical pattern of bactericidal activity, the Eagle effect, where the rate of killing decreases at increasing concentrations, has also been reported [23, 24]. On the other hand, as concentrations decrease, bacterial regrowth is observed. Against gram-negative bacilli, beta-lactams generally show either no persistent growth suppression or very short post-antibiotic effects (PAEs) [25], the carbapenems being the only exception in this class [26]. Therefore what is needed is an almost continuous supply of the antibiotic at concentrations high enough to produce an effect. The parameter that has been most used to assess the efficacy of time-dependent antibiotics is the time that the antibiotic plasma concentration exceeds the MIC for a particular microorganism, or time above MIC (t>MIC) [27-30].

The second group of antibiotics, which include the aminoglycosides and fluoroquinolones, exhibit a different killing pattern. In their case, the bacterial rate of killing is seen to increase with increasing concentrations of the antibiotic. The goal in this case is to maximize the drug concentration. The parameters that are most currently used are those which reflect an increase in drug concentration, i.e. Cmax/MIC (the ratio between the peak concentration and the MIC) and AUC>MIC (the portion of the area under the concentration time curve that is above the MIC). The AUC/MIC (or AUIC), area under the inhibitory curve is also popular and it can be easily calculated as the ratio of 24-hour AUC (for time points with respective concentrations above the MIC) and the MIC [28, 29, 31, 32]. Lately, the term AUIC is “out” and AUC/MIC is used.

t>MIC: Beta-lactams

This index measures the time during which antibiotic concentrations in plasma are above the MIC for a particular pathogen and it has been used to assess the bactericidal activity of β-lactam antibiotics [26, 33].

Craig has reviewed the data from the literature that use mortality as an endpoint and in which animals infected with S. pneumoniae were treated with penicillins or cephalosporins. A relationship was found between the duration of time that the serum level is above MIC and the efficacy, which was between 90-100% when serum levels were above the MIC for approximately half of dosing interval [34]. Maximal efficacy for cephalosporins in several animal infection models was approached when serum levels are above the MIC for 60%-70% of the dosing interval for Enterobacteriaceae and streptococci and for 40%-50% of the dosing interval for Staphylococcus aureus [35]. These results were confirmed by clinical studies in children with otitis media caused mainly by S. pneumoniae or H. influenzae. The authors suggest that in order to achieve approximately 80% of bacteriologic cure, serum concentrations of penicillins and cephalosporins should exceed the MIC of pathogens for at least 40% of the dosing interval [21, 36].

Cmax/MIC

This parameter is the relationship between the C_{max} reached in the patient at steady state and the MIC value established for the pathogen responsible for the infection [37]. Many studies have used it as a good predictor of clinical outcome for drugs showing concentration-dependent bactericidal effects, particularly for aminoglycosides [28, 37-40].

Moore et al. analyzed data from clinical trials of patients with gram-negative infections treated with gentamicin, tobramycin, and amikacin. They reported a strong correlation between clinical outcome (Figure 2) and a high C_{max}/MIC ratio for the aminoglycosides and suggest this parameter is a major determinant of the clinical response to aminoglycoside therapy [40].

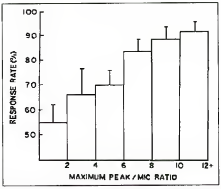


Figure 2: Relationship between peak level/MIC ratio and clinical response of aminoglycosides in 236 patients with gram-negative bacteremia [40].

To achieve a clinical response of >90%, the C_{max} should reportedly be 8- to 10-fold higher than the MIC [41]. Consequently, a once-a-day regimen has been proposed as the best administration mode for aminoglycosides [2, 42].

A consensus document that summarizes the advantages and provides guidelines for once daily dosing of aminoglycosides can be found in the Internet at www.boomer.org/pkin/consensus/Odacd.html.

Parameters that use AUC

The AUC above MIC (AUC>MIC) measures the area under the drug concentration-time curve in plasma at steady state for those points where concentrations are above the MIC. A modified version of this parameter, the AUC_{24h}/MIC ratio, relates the total AUC at steady state to the MIC, and has been found to best correlate with the efficacy of fluoroquinolones [2, 8, 31, 43]. The literature is somewhat confusing in regard to the terminology and sometimes AUC_{24h}/MIC and AUIC are used interchangeably. The term AUIC was introduced by Flaherty et al. for the area under the serum inhibitory concentration of clyndamicin. It was calculated (Figure 3) as the AUC of the reciprocal values of the serum inhibitory titer (SIT) vs. time, similarly to that previously described for evaluating the area under the bactericidal curve (AUBC) [44, 45].

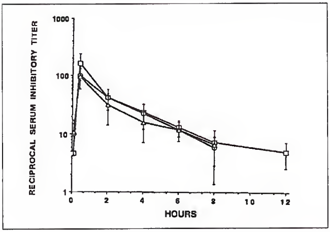


Figure 3: AUIC based on reciprocal serum inhibitory titers. Mean reciprocal serum inhibitory titers of clindamycin 600 mg q6h (♦), 900 mg q8h (□), and 1200 mg q12h (≤) against Bacteroides fragilis [45].

Later, Schentag et al. pointed out the equivalence between AUBC and AUIC for several antibiotic classes, and proposed a method to calculate AUIC as the AUC for the period of time the concentrations are above the MIC divided by the MIC itself (Figure 4) [8].

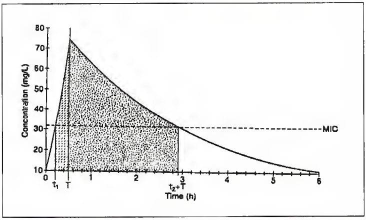


Figure 4: AUIC calculated as AUC/MIC. Simulated serum concentration-time profile of an antimicrobial drug assuming a one-compartment intermittent infusion model at steady state. T= infusion time; t_1=time when the MIC is reached during infusion; t_2= time after the end of the infusion when the concentration falls beloow the MIC. The shaded area represents the AUC above the MIC [8].

The same group has proposed that an AUIC value of at least 125 would correlate with bacterial eradication in about 7 days for both beta-lactams and quinolones, whereas an AUIC>250 would produce eradication by ciprofloxacin in approximately 1-2 days.

The authors state this value is also highly predictive of the development of bacterial resistance and that AUIC should be kept above 125 in order to prevent the onset of resistance. However, β-lactam antibiotics would still require longer treatment periods even at AUIC>250 due to their slower *in vivo* killing rate [8, 13].

The main problem of the AUIC is that it does not take into account the degree of fluctuation in the serum concentrations. Our group performed a systematic evaluation of the AUIC approach for three major antibiotic classes: β-lactams, quinolones and aminoglycosides. A precise equation for the calculation of AUIC was derived and different dosing regimens were simulated in order to attain the threshold AUIC=125 value. The equation for the most common case of a short-term I.V. infusion and a 1-compartment body model is shown below:

$$AUIC = \frac{k_0 \cdot N}{Cl \cdot MIC} \cdot \left[\frac{\left(1 - e^{-k(\tau - T)}\right)}{\left(1 - e^{-k\tau}\right)} \cdot \frac{\left(e^{-kT} - e^{-kt_1}\right)}{k} + T - t_1 + \frac{\left(e^{kT} - 1\right)}{\left(1 - e^{-k\tau}\right)} \cdot \frac{\left(e^{-kT} - e^{-k(t_2 + T)}\right)}{k} \right]$$

Equation 1

where k_0 is the infusion rate (mg/h), τ is the dosing interval, Cl is the total body clearance, k is the elimination rate constant, T is the duration of the infusion and t is the time elapsed since the start of the last infusion. The same AUIC value, which implies equal potency, was obtained with different dosage regimens that resulted in very different times above MIC (Figure 5). Particularly in the case of beta-lactams, the use of a constant AUIC value can produce regimens that have significant time below the MIC and may result in negative therapeutic effects [20].

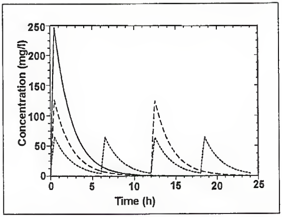


Figure 5: Simulation of the serum concentrations for three different cefmenoxime dosing regimens: 4215 mg q24h (solid line), 2135 mg q24h (long-dashed line) and 1039 mg q6h (short-dashed line). All 3 dosing regimens result in the same AUIC = 125 but in different therapeutic outcomes[20].

A study by Thomas et al. investigated the selection of bacterial resistance in relationship to antibiotic PK and organism MIC, through the development of PD models. Data from 4 clinical trials of lower respiratory tract infection by several microorganisms were studied. Patients were treated with cefmenoxime, ciprofloxacin, imipenem or ceftazidime. Microorganisms were divided into 4 groups according to their initial susceptibility. The final PK/PD model used was a variant of the Hill equation. The AUC_{24h}/MIC ratio was reported to be a significant predictor of resistance development, which was shown to be greater when $AUC_{24h}/MIC<100$ [46].

Disadvantages of the MIC approach

The MIC is a well-established laboratory parameter routinely determined in microbiology. It is currently by far the most commonly used pharmacodynamic parameter for the evaluation of efficacy of anti-infective agents. Once the "desirable" PK/PD parameters are determined, the drug concentrations are compared to the MIC to make dosing decisions.

However, although these parameters are useful predictors of the potency of the drug-microorganism interaction, this approach has both pharmacokinetic and pharmacodynamic disadvantages.

From the pharmacokinetic point of view, all of these models compare the MIC to measurements obtained from the concentration-time curve measured in plasma. Thus, two important factors are overlooked: protein binding and tissue distribution. After absorption most drugs are bound to plasma proteins. They can also leave the plasma and enter the tissues, where, again, they can be non-specifically bound to either tissue proteins or structures [47]. Protein binding is relevant because only the drug that is unbound from the plasma proteins will be available to exert a pharmacological effect. Tissue distribution also needs to be taken into account, given that most of the infections occur not in plasma, but in the interstitial space of the tissues.

From the pharmacodynamic point of view, the MIC approach does not provide any information on the kinetics of the drug action. For instance, the MIC value does not provide information on the rate of bactericidal activity and whether increasing antimicrobial concentrations can enhance this rate. Similarly, it does not provide any information about the persistent activity of the antimicrobial agent that remains following exposure to the drug [2].

Additional shortcomings are that MIC values are measured for constant antibiotic concentrations; therefore they are threshold values and do not reflect the *in vivo* scenario, where bacteria are being exposed to varying concentrations of the antibiotics due to the pharmacokinetic processes that take place in the body. Besides, they are measured in 2-fold increments; hence they are not very precise. Furthermore a threshold value implies the existence of an all-or-nothing concentration-effect relationship which does not reflect what is happening in real life. An additional source of error is the fact that the measurement is usually performed by visual inspection after overnight incubation (18-24 hours later) at one time point only.

Protein Binding

Knowledge of the protein binding is necessary to convert the total plasma concentrations to free plasma concentrations for comparison with the free tissue concentrations.

Kunin reviewed the effects of the protein binding on the antimicrobial activity of a series of β-lactam antibiotics (Figure 6) [48]. They showed that in *in vitro* incubation studies MICs are in good agreement with free non-protein bound and not with total drug concentrations, and concluded only the free drug in serum is available for antimicrobial activity [48-50]

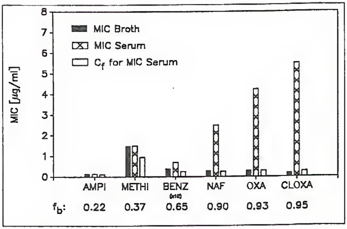


Figure 6: Effect of protein binding on antimicrobial activity. MICs of Staphylococcus aureus vs protein binding for several β-lactam antibiotics. AMPI= ampicillin, METHI= methicillin, BENZ= Benzylpenicillin G, NAF=nafcillin, OXA= oxacillin, CLOXA= cloxacillin, fb= fraction bound to protein. Bars: MICs measured in broth (black), in serum (black/white) and in serum corrected for protein binding (white) MICs[49, 50]

Although the MICs in serum seem to correlate to those in broth for drugs with low-bound drugs (Figure 6), it is evident from looking at the whole range of protein binding that this is not so. The MICs in serum are seen to increase with increasing protein binding while the MICs in broth stay at lower values. What this means is that the higher the protein binding, the more drug is going to be "sequestered" and therefore inactive.

Merrikin et al. confirmed these findings with study in mice where a direct correlation could be seen between the therapeutic activity *in vivo* and the extent of binding [51]. As a consequence, total plasma or tissue concentrations should not be considered because they include bound (inactive) drug together with the free drug, which is the only fraction active against the bacteria and thus the only one that is clinically relevant. Several articles have reviewed the factors that affect protein binding of antibiotics and its pharmacokinetic and therapeutic implications [52, 53]. Wise et al. examined the effect of protein binding on the penetration of β-lactams into tissue fluid using a cantharides blister technique. They found a linear relationship between the percentage of protein binding and the penetration into blister fluid, and concluded this was further evidence of the influence of protein binding on the efficacy of an antibacterial agent [54].

There are several methods for the determination of protein binding [55]. In the past, ultrafiltration has been used extensively [54-58]. More recently, determinations using microdialysis have been in good agreement with the traditional methods [59-63]

Although protein binding values have been reported in the literature, it is important to reassess them with the same plasma used for the assay [22, 64]. The plasma protein binding of cefaclor was determined by ultrafiltration in this study both for rat and human plasma.

Tissue Distribution

As explained above, only the free concentrations are available to exert an effect. Most infections occur not in the plasma but in the interstitial space of the tissues where the antibiotic free levels are most relevant.

The need for obtaining information on tissue penetration and concentrations is furthermore supported by the fact that even some antibiotics with a very low *in vitro* MIC fail to be effective in clinical studies, probably due to limited access to the target site. Once the free, therapeutically active concentrations of the antibiotic at the site of infection are known, they can be used to rationally adjust the dose of the antibiotics of interest [65-69].

A simple mathematical way to calculate free tissue concentrations of β-lactam antibiotics derived from conventional plasma pharmacokinetic data after I.V. bolus administration was derived by Derendorf. The concentrations predicted by these equations were found to be in agreement with clinical data [47].

Studies reporting tissue levels traditionally assay tissue homogenates, which is inaccurate because these represent the total drug in the tissue, both the active (free) and inactive drug. Another confounding factor is the presence of residues of drug-containing body fluids in the tissues (blood, urine) which would need to be removed beforehand [70]. Other approaches have been used over the years to try to determine tissue concentrations. Barza et al. investigated the penetration of antibiotics into fibrin clots and found that differences in penetration were directly related to the free concentrations in serum [71]. The pharmacokinetics of tissue penetration of antibiotics, mostly from animal studies, were reviewed by Bergan in 1981, with emphasis on the different models (i.e. skin blisters, tissue chambers, skin chambers, wound exudates, implanted fibrin clots, and peripheral lymph) available at the time [72]. Wise reviewed human studies of the influence of protein binding on tissue penetration. Advantages and shortcomings of various methods (tissue homogenates, synovial fluid sampling, wound fluid, skin chamber, skin blisters) were discussed [70]. In summary, several methods for the direct measurement of tissue concentrations of antimicrobial agents have been described (i.e. skin blister fluid method, saliva, or biopsy followed by tissue homogenization). However, they all share limits concerning their accuracy and feasibility, which are difficult to overcome in the clinical situation. In contrast to previously used techniques microdialysis selectively measures the unbound, active concentration of antibiotics in the interstitial compartment. Therefore, microdialysis provides the possibility to relate dosing regimens to the target tissue concentration at the site of infection [73-76].

The focus of this work is to determine the free, active concentration of cefaclor at the tissues that are its site of action. To date there is no report of free tissue concentrations of cefaclor by microdialysis after administration of different formulations and doses to human subjects. The thigh muscle was chosen due to its easy accessibility, and the same correlation found between muscle and lung in rats is assumed to exist in human subjects.

Microdialysis

Theoretical

Microdialysis is an analytical technique suitable for measuring the unbound fluid concentrations of the tissues and organs of the body. It is applicable to both animal and human studies. The most important features of microdialysis are that it allows sampling the extracellular fluid, and it simplifies chemical analysis by excluding large molecules from the perfusate. It has been extensively used in the neurosciences to monitor neurotransmitter release, and is now increasingly finding application in monitoring of the chemistry of peripheral tissues in both animal and human studies [77].

Microdialysis is based on the principle of dialysis. The driving force of molecular movement is diffusion down the concentration gradient existing between two compartments separated by a semi-permeable membrane. For sample collection, this membrane is continuously flushed on one side with an isotonic solution devoid of the substances of interest, while the other side faces the interstitial space. For the *in vivo* application of microdialysis, a concentration gradient is established between the extracellular fluid of the tissue and the artificial physiological solution flowing inside the membrane. If a probe is perfused with a physiologically composed perfusion medium and is inserted into the tissue of a subject that has been administered a drug, the drug will then diffuse passively from the extracellular fluid in the tissue to the perfusion medium in the probe [77-81]. Samples are then collected and analyzed.

The technique has several advantages for use in pharmacokinetic studies. First, after measuring low adenosine concentrations in the dialysate it has been reported that no major tissue trauma is induced by the catheter [82, 83]. Furthermore, the rapid concentration change of a low-molecular-mass compound detected in the dialysate following a change in the ambient concentration excludes interference by local concentration changes and edema [83, 84]. Although it is an invasive technique, histological examination of tissue microdialysed for 12 hours has shown that the tissue is intact without any major inflammatory reaction or bleeding adjacent to the microdialysis membrane [85, 86]. Microdialysis is also an economical method, because it provides the opportunity of obtaining several samples from one single animal or human subject. It has been suggested that the number of animals needed in preclinical pharmacokinetic studies can be substantially reduced by applying the microdialysis technique [87]. Because the sampling technique does not withdraw body fluid and, hence, does not disturb blood homeostasis, there are no limiting factors to the amount of samples taken from one animal. This not only minimizes the number of subjects necessary for a study as compared with terminal experiments, but it also minimizes the inter-subject variability as complete tissue pharmacokinetic profiles can now be obtained from a single subject. The technique has also the capability of sampling several tissues simultaneously. Finally, as the samples need to go through a semi-permeable membrane to be collected, they are already "filtered" and can be assayed directly after collection, without any pre-treatment.

In vivo microdialysis allows for simultaneous monitoring of unbound concentrations of the compounds in peripheral tissues. Microdialysis combined with accurate calibration is a reliable technique for studying the kinetics of drugs *in vivo* in different tissues. The utility offered by the microdialysis technique will have a bearing on future pharmacokinetic studies and it is to be expected that the importance of microdialysis in pharmacokinetic studies will grow in the future.

Recovery

Since the concentration in the dialysate will not fully equilibrate with that in the extracellular space, a parameter that needs to be determined in microdialysis studies is the probe recovery [80, 81, 88-96]. The ratio between the concentration of a substance in the dialysate and the concentration of the same substance in the tissue fluids is defined as recovery and expressed as a percentage. Retrodialysis is one way of calculating *in vivo* recovery. The probe inserted in the tissue is perfused with a solution containing a known concentration of the drug (C_{in}), and the dialysate (C_{out}) is analyzed. After equilibration, two samples of the dialysate are collected and the recovery is then calculated as $R = (C_{in} - C_{out})/C_{in} \cdot 100$ [97-99].

The no net flux method is another popular way of assessing recovery but it has the disadvantage of being more laborious and time consuming [82-85, 88, 89, 100, 101]. It is the most common method reference for assessing recovery and is based on the same principle as retrodialysis, with the addition of a continuous infusion that is administered to the investigated subject throughout the study. This infusion produces a constant concentration of drug in the tissues, and then solutions of different known concentrations of the drug are perfused and dialysate concentrations are measured. Depending on the concentrations in the perfusate (C_{in}) and the tissue (C_T), the diffusion direction will be from the probe to the tissue (if $C_{in}>C_T$), or from the tissue to the probe (if $C_{in}<C_T$). This will cause respectively a decrease or increase in the dialysate concentration (C_{out}) with respect to the perfusate. Finally the difference in concentration between the dialysate and perfusate ($C_{out} - C_{in}$) is plotted against the concentrations being perfused (C_{in}). The slope of the line represents the recovery and the point where the line crosses the x-axis (the point of no net flux, where $C_{out} = C_{in}$) represents the unbound tissue concentration.

Applications

Since its introduction in 1966, microdialysis has been extensively applied to studies in both animals and in men [102].

Animal studies

Previous studies with other beta-lactam antibiotics have indicated that rats are a suitable test system to investigate tissue distribution using microdialysis [66, 67, 103]. However, just one tissue has been studied at a time. This work intends to investigate the correlation between the concentration-time profiles in two different animal tissues simultaneously. The underlying assumption is that the same situation observed in rats can be extrapolated to human subjects.

The tissue penetration and distribution of antibiotics is of great importance, since most of the infections occur in the tissue. At the infection site, the free, unbound fraction of the antibiotic is responsible for the anti-infective effect. Microdialysis is a suitable method to evaluate unbound drug concentrations in the tissues.

Deguchi evaluated the extravascular equilibration and tissue binding mechanisms of β-lactam antibiotics. Concentrations of two antibiotics, cefminox and SY5555, in the lung, the muscle and the liver in rats were determined by microdialysis [103]. The dialysate concentration was extrapolated to the *in vivo* unbound concentration in tissue interstitial fluids according to an extrapolation method. This extrapolation method incorporates the effective dialysis coefficient of a reference compound, antipyrine, which is used to correct the difference between *in vivo* and *in vitro* permeabilities of microdialysis fiber. The unbound concentrations in interstitial fluid for cefminox and SY5555 in the lung, muscle and liver were close to the unbound concentrations in the venous plasma leaving these organs. Good correlation between the unbound concentrations of SY5555 in lung and muscle interstitial fluids estimated from the total concentrations in homogenized tissues and those extrapolated by the microdialysis studies was observed. This study also showed that total tissue concentrations differed widely between the tissues studied.

Plasma and tissue pharmacokinetics of ceftriaxone (high plasma protein binding up to 98%) in rats after single i.v. administration were investigated at two different concentrations (50 and 100 mg/kg) in our group [104]. Free tissue levels in the thigh muscle were measured by microdialysis. Both plasma and tissue samples were taken simultaneously and analyzed by reversed-phase high performance liquid chromatography (HPLC). Figure 7 shows the concentration in plasma is much higher than the free concentration in tissue. After determination of nonlinear protein binding by microdialysis and including these parameters in the pharmacokinetic model, this study indicated that it is possible to predict free concentrations in the interstitial space from plasma levels for any given dose.

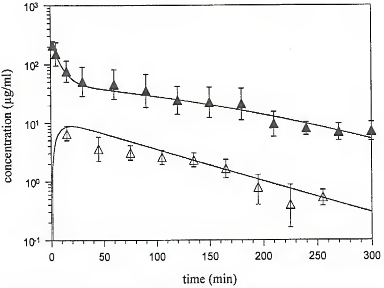


Figure 7: Concentration-time profiles (means ± SD) for ceftriaxone levels after i.v. bolus injection of 50 mg/kg [104]. Comparison of total plasma (▲) and free tissue levels (Δ). The tissue data are not fitted but the line shows the predicted levels based on the parameters obtained from plasma and protein binding data.

Our group has also used *in vivo* microdialysis sampling to study the tissue distribution patterns of piperacillin in anesthetized rats after single dose iv administration [66]. We also examined the relationship between free levels of piperacillin in blood with those in tissue. The pharmacokinetics of piperacillin in plasma were consistent with a two-compartment body model. Comparisons between calculated free concentrations in the peripheral compartment and measured free extracellular concentrations revealed excellent agreement. In case of piperacillin, predictions of the concentration time profiles of free drug in the peripheral compartment can be made on the basis of plasma data (Figure 8).

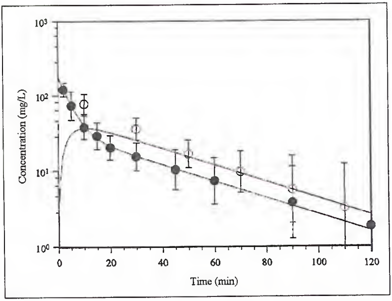


Figure 8: Concentration-time profiles for free piperacillin levels in plasma (●) and interstitial fluid (○) after administration of 60 mg/kg iv bolus combined with tazobactam [67]. Plasma concentrations were fitted to a two-compartment body model. The line for free tissue levels represents the prediction based on plasma data. Points represent mean ± SD of 12 animals.

A follow-up study was carried out to investigate the pharmacokinetics of piperacillin and tazobactam, alone and in combination after a single iv dose to anaesthetized rats [67]. Total plasma concentrations and free extracellular concentrations were quantified by HPLC. In-vivo microdialysis sampling was used to study the free tissue distribution patterns of both drugs. The pharmacokinetics of piperacillin and tazobactam in plasma were consistent with a two-compartment body model (Figure 9). Piperacillin pharmacokinetics were not influenced by co-administration of tazobactam. Tazobactam's volumes of distribution and clearance were decreased by the co-administration of piperacillin and the area under the curve was significantly increased. Comparisons between calculated free concentrations in the peripheral compartment for both drugs and measured free extracellular concentrations revealed excellent agreement.

For piperacillin and tazobactam, alone and in combination, predictions of the concentration-time profiles of free drug in the peripheral compartment can be made on the basis of plasma data.

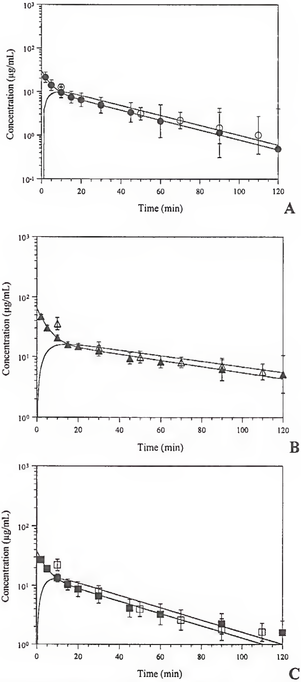


Figure 9: Concentration-time profiles of free tazobactam levels in plasma (filled symbols) and interstitial fluid (open symbols) after administration of 30 mg/kg (A, circles), 60 mg/kg (B, triangles) iv bolus and 30 mg/kg of tazobactam combined with 120 mg/kg of piperacillin (1:4) (C, squares) [67]. Plasma concentrations fitted to a

two-compartment body model. The line for free tissue levels represents the prediction based on plasma data. Points represent mean ± SD of 6 animals.

Microdialysis of intravenously injected theophylline in blood and in lung tissue was performed in two rats during anesthesia [91]. Since the recovery in blood was greater than the recovery in lung tissue and there was a change in recovery with time both in blood and in lung, an "internal standard", caffeine, was introduced to correct concentrations of theophylline in the dialysates. The corrected concentration of unbound theophylline in the blood was in accordance with the simultaneously measured total concentration of theophylline in plasma when binding to plasma proteins was taken into account. This study demonstrated that an "internal standard" for correction of the recovery is a useful method to approach the true concentrations of compounds in the extracellular water.

A microdialysis technique was applied to the *in vivo* intrabronchial pharmacokinetic measurement of tobramycin and gentamicin in the anesthetized rat [68]. The concentration versus time profiles of the drugs in the lung epithelial lining fluid (ELF) following intravenous bolus administration were determined. The results demonstrated that this technique can be used for intrabronchial pharmacokinetic measurement of drugs in the lung, either as an extension of the bronchoalveolar lavage technique or as a practical alternative to it.

Human Studies

The majority of the existing human studies have examined subcutaneous extracellular fluid concentration of endogenous agents. There are reports on the clinical use of microdialysis investigating tissue kinetics of antibiotics [69, 105], antitumor agents [106], and nicotine [107]. Some of these studies have investigated the kinetics of change of these agent when they are given exogenously [108]. For example, glucose, lactate, pyruvate or glycerol kinetics in subcutaneous adipose tissue have been studied after oral administration [109, 110]. The use of microdialysis in evaluating a number of human tissues e.g. skin, muscle, adipose, blood and brain, has been reviewed previously [80].

Microdialysis has become more and more popular in the studies of drug concentrations in peripheral tissues in humans. This technique allows the monitoring of metabolites and small molecules from the extracellular compartment as well as local delivery of metabolically active agents to this compartment. Cimmino et al. have recently reviewed practical issues (different types of probes, molecular cut-off, perfusion rate, dialysis buffer) and theoretical aspects of *in vivo* microdialysis (probe efficiency, recovery, perfusion rate, quantification of tissular metabolism) [94].

Recently, the microdialysis technique has been employed for the measurement of antibiotic concentrations in human tissues [69, 98, 111-113].

A study assessed the scope and limitations of the microdialysis technique for human tissue pharmacokinetic studies of acetaminophen and gentamicin [98]. Microdialysis probes were inserted into the medial vastus muscle or the periumbilical subcutaneous adipose layer of 13 healthy volunteers. Thereafter, volunteers received either acetaminophen (1000 mg orally) or gentamicin (160 mg intravenous bolus). Drug concentrations were monitored in plasma, muscle, and subcutaneous tissue. Calibration of the microdialysis probes was carried out *in vitro* and *in vivo* with use of the retrodialysis method. For both model compounds, complete concentration versus time profiles in muscle and subcutaneous tissue could be obtained. Major pharmacokinetic parameters (absorption half-life, elimination half-life, maximum concentration, time to reach maximum concentration, area under the curve, and area under the inhibitory curve) were calculated for tissues; tissue/plasma concentration ratios could be derived. Reproducibility of tissue drug concentration measurements was high.

In another study, microdialysis was applied to investigate the peripheral compartment pharmacokinetics of select antibiotics in humans [69]. Microdialysis probes were inserted into the skeletal muscle and adipose tissue of healthy volunteers and into inflamed and noninflamed dermis of patients with cellulitis. The volunteers received either cefodizime, cefpirome, fleroxacin, or dirithromycin; the patients received phenoxymethylpenicillin. For cefodizime and cefpirome, the AUCtissue/AUCserum ratios were 0.12 to 0.35 and 1.20 to 1.79, respectively. The AUCtissue/AUCserum ratios were 0.34 to 0.38 for fleroxacin and 0.42 to 0.49 for dirithromycin. There was no visible difference in the time course of phenoxymethylpenicillin in inflamed and noninflamed dermis. The authors suggest that the concept of pharmacokinetic/pharmacodynamic surrogate markers for evaluation of antibiotic regimens originally developed for serum pharmacokinetics can be extended to peripheral tissue pharmacokinetics by means of microdialysis. This novel information may be useful for the rational development of dosage schedules and may improve predictions regarding therapeutic outcome.

Müller et al. also examined whether free tissue levels of cefodizime (81% plasma protein binding) and cefpirome(10% plasma protein binding) in muscle and subcutaneous adipose tissue of healthy volunteers obtained by microdialysis are consistent with the extent of their respective plasma protein binding. Healthy volunteers were given both antibiotics at a single intravenous 2g dose in a randomized crossover design [114]. Microdialysis probes were inserted into a medial vastus muscle and into the periumbilical subcutaneous layer. In vivo recovery (34% and 39% for cefodizime and 30% and 33% for cefpirome, in muscle and subcutaneous tissue respectively) was assessed by retrodialysis. Drug concentrations were measured using a microagar diffusion bioassay. There was a reasonable agreement between plasma protein binding data and the tissue penetration of both cephalosporins into the interstitial fluid of the muscle tissue, but not for the subcutaneous tissue layer. Furthermore, the serum and tissue concentrations of both drugs were fitted to an open two-compartment body model. The measured free-tissue concentrations were compared with calculated unbound concentrations in the peripheral compartment. Good agreement was observed for the free muscle concentrations (Figure 10), but unbound concentrations in the subcutaneous tissue (Figure 11) was somewhat higher (cefpirome) or lower (cefodizime) than predicted. This may be due to the different lipophilicities of the two compounds.

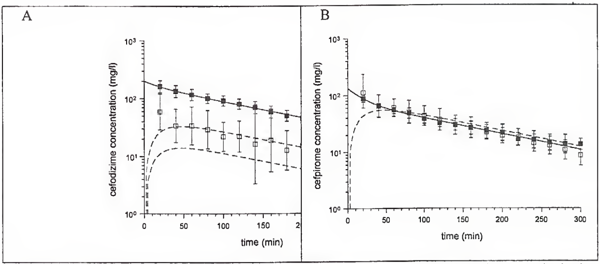


Figure 10: Concentration vs. time profiles of cefodizime (A) and cefpirome (B), respectively, in muscle tissue of healthy volunteers after iv. bolus injection of 2 g (geometric means ± S.D.) [114]. Total serum concentrations (—■—), unbound levels in muscle tissue (□) measured by microdialysis in comparison to the predicted range of unbound peripheral concentrations (--------) calculated from serum data and plasma protein binding (fraction bound for cefodizime ranged between 0.74 - 0.89, for cefpirome 0.10).

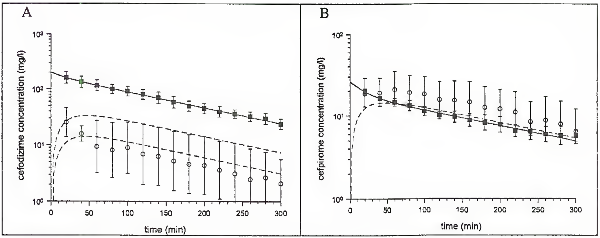


Figure 11: Concentration vs. time profiles of cefodizime (A) and cefpirome (B), respectively, in subcutaneous adipose tissue of healthy volunteers after iv. bolus injection of 2 g (geometric means ± S.D.) [114]. Total serum concentrations (—■—), unbound levels in subcutaneous adipose tissue (○) measured by microdialysis in comparison to the predicted time course of unbound peripheral concentrations (--------) calculated from serum data and protein binding (fraction bound for cefodizime ranged between 0.74 - 0.89, for cefpirome 0.10)

Interstitial ciprofloxacin concentrations in soft tissues were measured by microdialysis following intravenous administration to eight healthy volunteers [111]. In vivo probe calibration was performed by a retrodialysis procedure. Interstitial ciprofloxacin concentrations were significantly lower than the corresponding total serum drug concentrations; the interstitium-to-serum concentration ratios ranged from 0.55 to 0.73. An *in vitro* simulation based on interstitial pharmacokinetics showed a substantially lower antimicrobial activity than did the simulation based on serum pharmacokinetics. It indicates that ciprofloxacin concentrations at the site of effect may be subinhibitory although effective concentrations are attained in serum.

To characterize the penetration of moxifloxacin into peripheral target sites, unbound moxifloxacin concentrations in the interstitial space fluid were measured by means of microdialysis [112]. In addition, moxifloxacin concentrations were measured in cantharides-induced skin blisters, saliva, and capillary plasma and compared to total and free drug concentrations in venous plasma. 12 healthy volunteers received moxifloxacin in an open randomized crossover fashion either as a single oral dose of 400 mg or as a single intravenous infusion of 400 mg over 60 min. An almost-complete equilibration of the free unbound plasma fraction of moxifloxacin with the interstitial space fluid was observed, with mean area under the concentration-time curve (AUCISF/AUCPlasma ratios ranging from 0.38 to 0.55 and mean AUCISF/AUCFree Plasma ratios ranging from 0.81 to 0.86). The skin blister concentration / plasma concentration ratio reached values above 1.5 after 24 h, indicating a preferential penetration of moxifloxacin into inflamed lesions. The moxifloxacin concentrations in saliva and capillary blood were similar to the corresponding levels in plasma. The results show that moxifloxacin concentrations attained in the interstitial space fluid in humans and in skin blister fluid following single doses of 400 mg exceed the values for the MIC at which 90% of isolates are inhibited for most clinically relevant bacterial strains, notably including penicillin-resistant Streptococcus pneumoniae. These findings support the use of moxifloxacin for the treatment of soft tissue and respiratory tract infections in humans.

Skin blister fluid sampling, *in vivo* microdialysis and saliva sampling were compared as surrogates for the measurement of drug concentrations in peripheral compartments [75]. Paracetamol, a model drug with low protein binding, was administered to seven healthy volunteers at an oral dose of 2000 mg. Subsequently, tissue kinetics were measured simultaneously in cantharides induced skin blisters, microdialysates of subcutaneous- and skeletal muscle-tissue and saliva and compared to serum concentrations. Mean ratio AUCblister/AUCserum was 0.88, mean ratio AUCmuscle/AUCserum was 1.08, mean ratio AUCsubcutaneous/AUCserum was 0.96

and mean ratio AUCsaliva/AUCserum was 1.83. In this study the concentration profiles after single oral administration differed among the three methods. The time course of the concentration ratios showed that cantharides blister and microdialysate concentrations closely paralleled serum levels. An equilibration period of less than 2 h had to be taken into account for blister measurements. In contrast, saliva concentrations were significantly higher than serum concentrations. The results indicate that skin blister sampling and microdialysis closely mirrored corresponding serum concentrations and proved to be suitable techniques for the assessment of peripheral compartment pharmacokinetics. In contrast, saliva data overestimated the serum concentrations. Müller et al studied the tissue kinetics of theophylline following single dose administration simultaneously in cantharides induced skin blisters, saliva and microdialysates of subcutaneous- and skeletal muscle- tissue and compared to plasma concentrations [115]. Theophylline was administered to 9 healthy volunteers as an i.v. infusion of 240 mg. Mean ratio AUCsaliva/AUCplasma was 0.63, mean ratio AUCblister/AUCplasma was 0.69, mean ratio AUCmuscle/AUCplasma was 0.41, and mean ratio AUCsubcutaneous/AUCplasma was 0.34. The time course of the concentration ratios showed that tissue concentrations obtained by microdialysis were closely correlated to free plasma levels, whereas saliva- and cantharides blister data overestimated the corresponding free plasma concentrations. It is concluded that microdialysis represents a reliable technique for the measurement of unbound peripheral compartment concentrations and is superior to saliva- and skin blister concentration measurements.

The presented examples illustrate the explosion of new research in the application of microdialysis to measure both endogenous as well as exogenous compounds in peripheral tissues. For many years, pharmacokinetics was limited to blood and plasma concentration measurements. Although it was always well known that the site of action for most drugs is in the peripheral tissues, vascular concentrations were measured and therapeutically interpreted, simply because they were easy to obtain. Total tissue concentrations based on homogenized tissue biopsies further confused the situation. These 'concentrations' which are quite commonly obtained in toxicology studies are not very helpful since they reflect hybrid concentrations of free and bound drug and cannot easily be interpreted correctly. Microdialysis measurements in peripheral tissues has solved this ambiguity and opened the window to direct measurement of the unbound drug concentrations of interest at the site of action. It can be expected that this technique will be further refined during the next years and will find a standard place in the arsenal of pharmacokinetic evaluation tools.

PK/PD Models Based on Kill Curves

A different approach to assess the anti-infective efficacy of antibiotics is to use PK/PD models based on time-kill curves. Time-kill curves can follow microbial killing and growth as a function of both time and antibiotic concentration. Antibiotic concentration can either be held constant or changed to mimic an in-vivo concentration profile, be it either in plasma or tissues. The resulting kill curves can be subsequently analyzed with appropriate PK/PD models. Finally these PK/PD models would allow to optimize dosage regimens based on a rational, scientific approach. The advantage of these in-vitro models is that they allow direct comparison of various concentration profiles and provide for a much more detailed assessment of the PK/PD relationship than the simple use of MIC values.

Kill curves can and have been used to study anti-infective effects both in animal and *in vitro* models, with the advantage of providing more detailed information about the time course of antibacterial effect.

In Vitro Models

Various types of *in vitro* models have been devised. The main classes are:

1) those with constant antibiotic concentrations, which study the effects of a constant concentration of drug against bacteria as a function of time; and
2) those with variable antibiotic concentrations, in which the antibiotic concentrations are made to fluctuate by dilution or diffusion

Models with constant antibiotic concentration

These models study the number of bacteria exposed to a constant antibiotic concentration. Early studies by Garrett et al. investigated the effect of constant concentrations of bacteriostatic and bactericidal drugs against bacteria as a function of time [116-118].

Curves in the presence (kill curves) and absence (growth curves) of antibiotic were compared. After an initial lag time, bacteria typically show a phase of logarithmic growth (log phase) that can be described by:

$$N = N_0 \cdot e^{-k_0 t}$$

Equation 2

where N is the number of bacteria at any given time point, N_0 is the number of bacteria in the initial inoculum and k_0 is the first-order growth rate constant. N and N_0 are usually expressed in colony forming units per mL (CFU/mL). Exposure to an antibiotic while the bacteria are in log phase will produce a change in k_0, resulting in a different growth rate constant (k_{app}).

The mathematical relationship between antibiotic concentration and k_{app} for bacteriostatic drugs was studied, and the interactions found were classified into four classes by Garrett [119]. The classes were defined as follows:

1) Class I interactions are defined by a linear relationship between k_{app} and concentration that can be expressed by:

$$k_{app} = k_0 - k_i \cdot C$$

Equation 3

where k_I is a second-order inhibitory rate constant specific for the drug, C is the drug concentration, and k_0 and k_{app} have been defined before.

2) Class II interactions, where the k_{app} decreases with increasing concentrations to approach zero asymptotically, are represented by the equation:

$$k_{app} = k_0 - \frac{K_A \cdot C}{1 + K_B \cdot C}$$

Equation 4

where K_A is an equilibration constant between the medium and the receptor sites at steady state, K_B is the affinity constant between drug and receptor, and C, k_0 and k_{app} have been defined before.

3) Class IIII interactions exhibit Class I behavior at low concentrations, which turns into Class II behavior at high concentrations.

4) Class IV interactions show S-shaped plots of k_{app} vs. concentration that may be due to binding of the drug to nutrients at low concentrations.

The main difference between bacteriostatic and bactericidal drugs is that for the latter the k_{app} may take negative values, as the bacteria are now being killed. There is a linear relationship between k_{app} and C that can be expressed mathematically by:

$$k_{app} = k_0 - k_i \cdot (C - C_i)$$

Equation 5

where k_0 is the growth rate constant in absence of drug, k_i is the kill rate constant, C the drug concentration and C_i the minimum drug concentration that will produce an effect [120].

Using a different mathematical approach, the growth curves for bacteria exposed to different antibiotic concentrations were described taking into account the effect lag time after initial drug exposure. Curves of growth in the presence of the antibiotic were expressed as quadratic functions of time, with initial growth rates and rates of change of growth as concentration dependent variables. Assuming k_0 is a constant, kill rates would only depend on drug concentration [121-124].

A disadvantage of these approaches is that they do not reflect the *in vivo* situation where drug concentrations fluctuate. Therefore, although they are good mathematical descriptors of antibacterial behavior, they have limited ability of predicting or correlating with clinical outcomes.

Models with changing antibiotic concentrations, on the other hand, try to simulate *in vivo* concentration-time profiles using human PK parameters in order to assess the antibacterial effect. Changing concentrations can be produced either by dilution or diffusion.

Models with variable antibiotic concentration

Dilution models. A kinetic model that operated by pumping sterile broth into a flask (containing bacteria in log growth phase and antibiotic) at a constant rate resulted in antibiotic dilution [125, 126]. The main limitation of this model was that the volume in the flask increased throughout the experiment, diluting also the inoculum and posing practical problems for large dilutions. A one compartment open model that used a system consisting of 2 flasks interconnected by a peristaltic pump to simulate first-order kinetics was developed by Grasso et al [127]. Sterile broth was pumped from the diluent reservoir flask to the flask containing the bacterial culture and the antibiotic. The volume in the culture flask could be regulated, resulting in exponentially decreasing antibiotic concentrations that simulated the human serum $t_{1/2}$ of the antibiotic. An adjustment in the model enabled it to also simulate first-order absorption kinetics [127]. Further modifications of this model enable to continuously monitor bacterial level by including a photometer to read turbidity levels, and to control the system using a computer [128-131]. The main concern with these models is that the bacterial inoculum is diluted together with the antibiotic. This effect is particularly important when the dilution rate is higher than the bacterial growth rate, and the resulting increased bacterial clearance needs to be accounted for. Some equations to correct this effect for exponentially changing cultures have been proposed [132, 133]. Löwdin et al. employed a 0.45-µm filter membrane in the culture flask to prevent bacterial outflow. By placing a stirrer above the filter, they avoided blockage of the membrane and ensured that the culture was homogeneously mixed. The culture flask had two arms, one of which was used to take samples through a silicon diaphragm. Media was drawn from the culture vessel by a pump at a constant rate, causing a vacuum in the flask that in turn caused fresh sterile media to be sucked into the flask through the other arm. The antibiotic was added through the first arm and followed first-order elimination kinetics [134]. The original model contained the stirrer with a magnet covered in Teflon®, however, bacteria were suspected to adhere to this surface. The Teflon-covered stirrer was substituted by a new stirrer in which the magnet was encased in glass [135]. A modification of this model allows to study the effects of antibiotics on intracellular pathogens [136]. Instead of the culture flask, a glass chamber with a metal rack fitting Falcon cell culture inserts was used and connected to a pump. Cells cultures are grown in the inserts, infected and then transferred to the glass chamber, which contains media with antibiotic. Half-lives of the antibiotics are simulated using a pump as described above, and samples are taken at appropriate time points. A magnet is placed in the bottom of the glass chamber to achieve an even concentration through the medium. After the experiments, cells are lysed and intracellular bacteria counted by plating [136].

Diffusion models. In diffusion models, the antibiotic concentrations are changed by applying concentration gradients. An in-vitro model in which the compartments were separated by a hollow-fibre dialyser was used to simulate 2-compartment kinetics. Drug dilution was achieved by pumping sterile broth from one compartment to the other. However, the model was reportedly too complex and impractical. Its use was recommended only when a very short drug $t_{1/2}$ needed to be simulated or very flexible kinetics required [137]. A different 2-compartment model using a regenerated cellulose dialyser was developed by König et al [138, 139]. Dilution was accomplished by antibiotic diffusion from the culture compartment to the second compartment along a concentration gradient.

A model by Murakawa et al. uses bi-directional flow between the central and peripheral compartments to achieve a bi-exponential decline in concentrations. Bacteria and drug are placed initially in the central compartment, broth is pumped into the central compartment and bacteria are diluted out of the central compartment and the system, therefore necessitating mathematical adjustment for bacterial dilution [140]. A model that integrates bi-directional flow with the use of a membrane to prevent bacterial dilution has been used by Palmer et al [141].

A more complex model to simulate 2-compartment PK was introduced by Blaser et al [142]. Serially placed bacterial compartments, representing extravascular infection sites, interface with a central compartment through artificial capillaries. Broth containing antibiotic is constantly pumped from the central compartment through the tubing. The porous capillary walls allow for bi-directional passage of antibiotics (<10 kD) but are impermeable to bacteria. Sterile drug-free broth is continuously pumped to the central compartment from a diluent reservoir, thus displacing drug-containing broth from the system. An additional "absorption" compartment may be added between the diluent reservoir and the central compartment to simulate first-order absorption [41]. The model can be used for simulation of both continuous and intermittent drug administration [143]. Simultaneous first order elimination kinetics of two drugs with different half-lives were simulated to study antibacterial effects of drug combinations [144-146].

In vitro models to simulate *in vivo* conditions in specific infection sites or conditions, such as an bladder [147, 148], bacterial cystitis [149], otitis media [150, 151], endocarditis [152], chronic pneumonia [153], tuberculosis [154], infected fibrin clots [155], and implant-related infections [156, 157] have been developed.

Our group developed a simple one-compartment *in vitro* model to study the PD effect of concentrations of piperacillin against E. coli, following administration of constant or fluctuating concentrations [158]. The free interstitial concentrations of the drug reached in humans after different doses and dosing regimens were simulated. To simulate drug elimination in a stepwise fashion, broth solution containing antibiotic was withdrawn from the model at fixed time intervals and replaced with drug-free broth. The bacterial inoculum was not changed because the broth was both withdrawn and added through a filter. The number of cells was determined by colony count after overnight incubation. This model was also used in a separate study for the determination of the effect of different dosing regimens of piperacillin alone and in combination with tazobactam on E. coli [159]. The same *in vitro* model will be used in this project for the evaluation of the effect of various dosing regimens of cefaclor on four different bacterial strains.

Animal Models

Several animal models that have been used in the past years to evaluate the PD of different antibiotics are briefly reviewed below.

Zhi et al. published the mathematical solutions for two possible PD interactions (linear nonsaturable and nonlinear saturable) between β-lactam antibiotics and microorganisms [160]. The equations, developed from a model originally proposed by Jusko et al. [161] were derived for different dosage regimens, such as single and multiple I.V. bolus, constant infusion at steady state. The authors applied the model to the activity of piperacillin against P.aeruginosa in neutropenic mice and concluded that the saturable nonlinear model appeared to be appropriate. Relevant equations are briefly shown under "PK/PD data analysis" [160].

Craig et al. studied the pharmacodynamics of amikacin against gram-negative bacilli and the impact of the dosing regimen (τ) on the efficacy of amikacin in thigh-infection and pneumonia models, in mice with normal and impaired renal function [28]. The studies in animals with renal impairment were designed to simulate the PK profile of amikacin in humans. Mice were rendered neutropenic by cyclophosphamide injection, renally impaired with uranyl nitrate and lung and thigh infections were induced. Animals were treated for 24 hours with amikacin total doses ranging from 1.5 to 3072 mg/Kg, administered either as a single dose or every 6 or 12 hours. Treatment was started 2 or 14 hours after thigh or lung infection, respectively. Four to seven serial 2- or 4-fold increases in total dose were used for each organism to characterize the complete dose-response curve from no effect to maximum effect. Animals were killed at several time points up to 24 hours (normal renal function) or 48 hours (renally impaired). Lungs and thighs were removed, homogenized and plated on agar for bacterial count determination. In vivo antimicrobial activity was characterized by a sigmoid dose-response model previously described by Leggett [28, 31].

The authors observed amikacin $t_{½}$ were closer to human values and post-antibiotic effects (PAEs) were longer in neutropenic, renally impaired mice. In the thigh-infection model, the E_{max} and maximum bactericidal effect (defined as the maximum reduction in CFU from the starting inoculum) were greater in renally impaired mice than in normal mice; while in the pneumonia model, they were relatively similar in both groups of mice. This was largely due to the greater killing of K. pneumoniae in the lungs of normal mice, compared to the thighs. As τ increased from 6 to 24 hours, P_{50} tended to increase in mice with normal renal function, but this had little impact on the P_{50} values for amikacin in mice with impaired renal function. The authors conclude that prolonged PAEs under PK conditions similar to those observed in humans minimize the impact of τ on efficacy of amikacin and accentuate its concentration-dependent properties [28]. Several studies have used the thigh infection model [33, 162-167] as well as the pneumonia model [168-171].

An experimental model of endocarditis has been extensively used to study the pharmacodynamics of β- lactams [17, 172-176], aminoglycosides [176], fluoroquinolones [177] and glycopeptides [178] in either rabbits or rats, including the effect of sub-bactericidal concentrations [179]. In a review by Andes and Craig, the magnitudes of the PD parameters required to achieve efficacy in studies of experimental endocarditis for fluoroquinolones were observed to be similar to those observed in the murine thigh infection model [177]. An intraperitoneal infection model has been used to study β-lactams and aminoglycosides [180-182] and glycopeptides [183] in mice or rats, either neutropenic or not. Models for specific infections include systemic infection [160] device-related infection [157] and mastitis [184].

Clinical Studies

The validity of several PK/PD indices predictive of antibiotic efficacy (AUC/MIC, AUC>MIC, AUIC, t>MIC) was recently evaluated using clinical data reported for ciprofloxacin and different correlation models [7].

Currently there are very little clinical trial data available to specifically correlate the PK of the free drug in its site of action to its PD parameters following a kill-curve approach [113]. Drusano reviewed the literature correlating PK of antibiotics and clinical outcome, and suggested that for β-lactam antibiotics, t>MIC is the will have a direct relationship to patient outcome from serious bacterial infections [27, 185]. Most of the available literature focuses on the purely pharmacokinetic aspects of the drug, describes clinical improvement according to symptoms, or uses an MIC-based approach to describe the pharmacodynamics [46, 186-190].

PK/PD Data Analysis

For an antibiotic showing nonlinear saturable drug-receptor interaction, the kinetic equation of the rate of change in quantity of bacteria with time is represented by:

$$\frac{dB}{dt} = kapp \cdot B - \frac{k'_{kill} \cdot C}{K_m + C} \cdot B$$

Equation 6

where k_{app} (min^{-1}) is the apparent growth rate constant of the organism, B is the bacterial concentration in CFU/mL, C is the antibiotic concentration (μg/mL), K_m is a Michaelis-Menten type constant (μg/mL) and k'_{kill} (min^{-1}) is a bacterial killing rate constant.

Single I.V. dose. Substitution of C for the expression for I.V. bolus concentration: $C = C_0 e^{-ke \cdot t}$, and subsequent rearranging and integration yields:

$$\log f = \frac{k_{app} \cdot t}{2.3} + \frac{t_{1/2} \cdot k'_{kill}}{2.3 \cdot \ln 2} \cdot \ln\left[\frac{K_m + C_0 (1/2)^{t/t_{1/2}}}{K_m + C_0}\right]$$

Equation 7

where f is the bacterial survival fraction equal to B/B_0, B_0 is an initial bacterial concentration just prior to antibiotic administration, and $t_{1/2} = \ln 2/k_e$. The authors postulate that for a given antibiotic (following a 1-compartment PK model but a nonlinear PD model), dose, and microbiological species, Equation 7 allows interpreting microbial death and growth under the influence of changing antibiotic concentration after single administration. By further manipulation of Equation 7, the optimal time to administer the next dose and the minimum critical antibiotic concentration in blood can be calculated.

Multiple dosing. For multiple dosing, the term C in Equation 6 is substituted by:

$$C = C_0 \cdot \gamma \cdot e^{-k_e \cdot t'}$$

Equation 8

where t' is the time within a dosing interval and γ is a pharmacokinetic accumulation factor:

$$\gamma = \left(\frac{1 - e^{-N \cdot k_e \cdot \tau}\,.}{1 - e^{-k_e \cdot \tau}\,.}\right)$$

Equation 9

where N is the number of doses of the multiple dosing regimen and τ is the dosing interval. The resulting final PD equation is:

$$\log f = \frac{k_{app} \cdot t'}{2.3} + \frac{t_{1/2} \cdot k'_{kill}}{2.3 \cdot \ln 2} \cdot \ln\left[\frac{K_m + \gamma \cdot C_0 (1/2)^{t'/t_{1/2}}}{K_m + \gamma \cdot C_0}\right]$$

Equation 10

Steady-state. Substitution of C for C_{ss} in Equation 6 yields the equation for the rate of change of bacteria at steady-state drug concentrations (C_{ss}):

$$\frac{dB}{dt} = kapp \cdot B - \frac{k_{kill}^{'} \cdot C_{ss}}{K_m + C_{ss}} \cdot B$$

Equation 11

which after appropriate rearrangement and integration yields:

$$\log f = S' \cdot t = \left[\left(k_{app} - \frac{k_{kill}^{'} \cdot C_{ss}}{K_m + C_{ss}} \right) \Big/ 2.303 \right]$$

Equation 12

where S' is the slope of the linear curve of log f vs. time t [160].

Firsov et al. used a two-compartment *in vitro* dynamic model with antibiotic and bacterial dilution to study the effect of antibiotics and suggested two integral parameters to characterize antimicrobial effect duration (T_E) and intensity (I_E). The first parameter, T_E is defined by the time from the moment of antibiotic administration to the moment when the bacterial count reaches its initial level again, and I_E is the area between the microbial growth curves in the presence and absence of an antibiotic [191]. These parameters were compared to 10 other indicators of bacterial killing and regrowth kinetics in terms of the respective AUC-response relationships as applied to the action of ciprofloxacin against E. coli. Comparisons were made on the basis of 4 criteria: relevance to be related to the AUC, sensitivity to the AUC, robustness and predictive ability in terms of I_E. The authors conclude only I_E and T_E met all 4 criteria and propose I_E as a more universal measure of the antimicrobial effect [192]. Using the same model, commonly used predictors of antimicrobial effect (AUC/MIC, AUC>MIC, t>MIC) were examined for pharmacokinetically different quinolones, trovafloxacin and ciprofloxacin. Linear correlations were established between I_E and log AUC/MIC, log AUC>MIC and log t>MIC, being the first two specific for each drug and dosing regimen, thus allowing for quantitative comparison of the effects [193]. The efficacies of trovafloxacin and ciprofloxacin against gram-negative pathogens were studied and linear relationships were established between I_E and AUC/MIC by the following equation:

$$I_E = a + b\log(AUC / MIC)$$

Equation 13

To express the antimicrobial effects as a function of the quinolone dose (D), the term AUC in Equation 13 is substituted for:

$$AUC = c + dD = eD^2$$

Equation 14

The values for c, d, and e were obtained from AUC-D plots constructed from reported data [194]. Recent studies by the same group have applied the same concepts to study different bacterial strains [195] and to investigate the inoculum effect for quinolones [196] and β-lactams [197].

The following sigmoid dose-response model was used by Craig et al. to characterize the *in vivo* antimicrobial activity in an animal model, which is described above.

$$E = \frac{E_{\max} D^n}{P_{50}^n + D^n}$$

Equation 15

where E is the observed effect at 24 hours (measured as the reduction in log10 CFU/thigh or lung compared to untreated controls), D is the cumulative 24-hour dose of amikacin, E_{max} is the maximum antimicrobial effect attributable to the drug, P_{50} is the 24-hour dose producing 50% of E_{max}, and n is the slope of the dose-response relationship. E_{max} was calculated from the change in $\log_{10}$ CFU/thigh or lung over the 24 hours of treatment, at the highest dose studied for each organism. P_{50} and n were calculated separately for each dosing interval using non-linear regression.

Continuous Administration of Beta-lactam Antibiotics

The goal of the therapy with time-dependent killing antibiotics is to maximize the time the drug, at levels higher than the MIC, is in contact with the bacteria [186, 198]. In order to achieve this goal, various dosing strategies have been devised: drugs can be either given as a continuous infusion, or they can be administered frequently at short time intervals [25, 26, 199-202]. New strategies for improving antimicrobial therapy have suggested the use of continuous infusion as a way of administering β-lactam antibiotics in order to achieve the most benefit with the least amount of drug [198, 203]. Those with shorter half-lives would be the best candidates for this type of administration, whereas intermittent administration would be preferred for beta-lactams with longer half-lives. A couple of additional benefits from the administration of continuous infusions is that reduced doses may be sufficient to achieve the desired effect, ultimately resulting in diminished health care costs and potentially reducing the risk of toxicity [202, 204]. A consensus document that summarizes the advantages of continuous administration of antibiotics can be found in the Internet [26]. However, the administration of continuous infusion also has some disadvantages: it is not too convenient from the patient's point of view, whose mobility is restricted by the need of constant I.V. access. Continuous venous occupation may be problematic if several medications or fluids need to be co-administered, raising concerns about compatibility and stability issues. Last but not least, if the concentrations produced by the infusion are constantly too close to the MIC of the pathogen of interest, selection of resistant strains is a possibility, and could result in adverse clinical outcomes [26, 202]. The efficacy of continuous infusions of beta-lactam antibiotics as compared to intermittent injections has been studied in animal models [17, 172, 201, 204-207]. In general, continuous infusion of penicillins and cephalosporins has been shown to be superior to intermittent doses against both gram-positive and gram-negative infections, particularly after complement depletion and granulocytopenia. However, experimental infection studies with gram-negative cocci have yielded conflicting results as to whether continuous infusions would be preferable to intermittent dosing regimens. Nevertheless, continuous infusion of β-lactams consistently exhibits more potency than intermittent administration for enterobacteria and Pseudomonas aeruginosa. Similar results were observed in studies of β-lactams in combination with aminoglycosides [26].

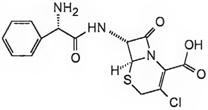
In humans, administration of continuous infusions has resulted in good clinical efficacy in various cases, including neutropenic patients that did not respond to intermittent dosing and cystic fibrosis patients infected with Pseudomonas aeruginosa [26, 208, 209]. Following a sequential clinical study protocol, Zeisler et al. determined that a loading dose of cefuroxime followed by continuous intravenous infusion provided shorter length of treatment and hospital stay, lower total dose, and cost savings when compared to intermittent I.V. piggyback infusion. They showed the use of continuous infusion of cefuroxime in humans to be safe, practical, useful and cost-effective [26, 210]. In summary, although further clinical trials would be needed to confirm the available data, the results of many *in vitro* an animal studies, as well as a few case studies and clinical trials suggest that continuous infusion may be the optimal method of β-lactam administration. This method has shown many potential microbiologic, clinical and economic advantages, without serious contraindications. Such therapy might enhance the efficacy of these antibiotics against some organisms, while reducing treatment costs [26].

Another possible approach to the same therapy goal is to administer the drug in an extended release formulation. Using the modified release approach, drug concentrations may be maintained at effective levels for a longer period of time, without the inconveniences to the patient derived of the need of venous access. This is particularly useful in the case of beta-lactam antibiotics with a short half-life that would otherwise need to be administered at very frequent intervals.

Cefaclor

Pharmacokinetics

Cefaclor (CCL), 3-chloro-7-D-(2-phenyl-glycinamido)-3-cephem-4-carboxylic acid, is a semisynthetic, orally available, broad-spectrum cephalosporin antibiotic [211-213] whose chemical structure is shown below.



Cefaclor (M.W.: 367.81)

Cefaclor is a second-generation oral cephalosporin. Cephalosporins of the second generation are characterized by a greater potency against gram-negative bacteria, like β-lactamase producing strains of Haemophilus influenzae and Moraxella catarrhalis. The drug also displays features of a first generation cephalosporin. First-generation cephalosporins are generally more active against gram-positive organisms than the second- or third-generation cephalosporins. Gram-positive coverage includes nonpenicillase- and penicillase-producing Staphylococcus aureus (methicillin-resistant strains are resistant) and Streptococci (except Enterococci). Gram-negative coverage includes Haemophilus influenzae, Moraxella catarrhalis, Escherichia coli, Klebsiella species and Proteus mirabilis. Clinically it is used primarily to treat otitis media, sinusitis, and upper respiratory tract infections. In controlled-clinical trials over a period of twenty years, enrolling more than 18.000 patients, including children and elderly adults, cefaclor showed a convincing therapeutic efficacy in a variety of community-acquired infections [211]. Cefaclor is well tolerated. 90% of patients in clinical trials did not experience any adverse drug reactions. Most frequently reported adverse drug reactions include hypersensitivity (about 1.4%) and diarrhea (about 1.3%). An isolated anaphylactic reaction in a pediatric patient was recently reported [214]. In general, adverse drug reactions are mild to moderate in nature and either self-limited or subside upon cessation of drug exposure. Cefaclor was approved by the FDA in 1979.

Absorption. Cefaclor is well absorbed from the gastrointestinal tract. Following oral administration in healthy, fasting adults, peak concentrations of cefaclor are attained within 30-60 minutes and average 5-11 μg/ml following a single 250 mg dose, 8-17 μg/ml following a single 500 mg dose and 25-35 μg/ml following a single 1 g dose [215-220]. It could recently be shown that the absorption mechanism involves a proton-dependent dipeptide transport carrier located in the brush-border membrane of the small intestine enterocyte [221, 222]. This active carrier has been well characterized in human Caco-2 cell cultures where uptake was saturable and competitively inhibited by coadministration of other dipeptides including β-lactam antibiotics. Food intake reduces the maximum cefaclor concentrations (C_{max}) and prolongs the time of the maximum concentration (t_{max}) [215, 223]. In a study of 12 healthy male volunteers, the mean cefaclor Cmax-values after an oral dose of 250 mg were 8.7 μg/ml in the fasted state and 4.3 μg/ml after breakfast [215]. The respective mean t_{max} values were 0.6 h and 1.3 h. However, the mean areas under the curve for both groups were 8.6±1.4 μg/ml·h under fasting conditions and 7.6±1.2 μg/ml·h after breakfast and not significantly different from each other. This indicates that the extent of absorption of cefaclor is not altered by food. In another study the effect of different kinds of food on cefaclor absorption was studied [223]. Cefaclor (500 mg) was given to eight volunteers at five test times: after overnight fasting, after two rice meals with bean paste soup, pickles and milk (350 and 700 cal) and after two bread meals with margarine, egg and milk (500 and 1000 cal). Area under the curves and urinary recoveries of cefaclor were not affected by the various meals, but C_{max} was reduced and t_{max} was prolonged depending on the type and the quantity of the meal. The larger the quantity of the meal, the more the maximum concentration of drug in plasma and the time to maximum concentration were affected. The average C_{max}-values were 14.8 μg/ml (fasted), 9.1 and 5.9 μg/ml (rice meals with 350 and 700 cal) and 7.9 and 6.8 μg/ml (bread meals with 500 and 1000 cal).

Absorption of cefaclor can be sustained using appropriate oral dosage forms such a cefaclor advanced formulation (cefaclor AF) which increased t_{max} from 1.5 h (immediate release) to 3.1 h (cefaclor AF) [224]. Total bioavailability was not altered, the respective AUC values were 20.5 vs. 22.1 μg/ml·h, respectively.

As most β-lactam antibiotics, cefaclor is absorbed by an active transport mechanism involving a dipeptide carrier. Since this carrier is located in the upper part of the small intestine, this results in an 'absorption window' limiting the surface area available for cefaclor absorption.

Distribution. Cefaclor exhibits negligible plasma protein binding of approximately 20-25% [22, 64]. However, in another study, a plasma protein binding of approximately 50% is reported [225]. Cefaclor does distribute into sputum [226]. Sputum levels in patients were 8-10% of the serum levels and high enough to inhibit the growth of Pneumococci, other Streptococci and Meningococci. The mean peak sputum concentrations were 0.4 and 0.6 μg/ml for the doses of 0.5 g and 1.0 g, respectively. In another study, tissue penetration was studied using skin blister fluid [227]. After oral doses of 250 and 500 mg, plasma peak levels of 10.6 and 17.3 μg/ml were measured. The respective C_{max} in blister fluid were 3.6 and 6.5 μg/ml. However, comparison of the areas-under-the curve shows that the AUC in blister fluid was 89% and 82% of the AUC in plasma.

After an oral dose of 500 mg cefaclor, peak concentrations in gingiva were measured to be 3.7 μg/g and concentrations in mandibular bone 1.6 μg/g [228]. At the same time, peak plasma concentrations of 7.6 μg/ml were measured. Although in this study, the measured tissue concentrations compared favorably with respective minimum inhibitory concentrations (MICs) such a comparison should be done with care since tissue concentrations reflect average total amount of drug per amount of tissue, hence the sum of free and bound drug. It is only the free drug, however, that will be able to produce the anti-infective effect.

The average peak concentration of cefaclor in aqueous humor is 0.7 μg/ml and occurs three hours after a 1 g oral dose [64]. This concentration does not approach the MIC of most organisms that are commonly responsible for bacterial endophthalmitis. Although *in vivo* data appear to be lacking, cefaclor presumably does not cross the blood-brain barrier well [64].

Metabolism. Cefaclor shows chemical instability and degrades to presumably inactive products [229, 230]. In vitro at 37° C, only 10% of the original microbiologically active form is found in plasma after six hours [230]. This is equivalent to a half-life of approximately two hours. The metabolic fate of oral cefaclor has been studied in rats, mice and dogs [212]. In rats and mice, cefaclor, for the most part, escapes metabolism in the body and is eliminated unchanged as unaltered antibiotic. In dogs, however, cefaclor is more readily metabolized and only a portion of the administered antibiotic is eliminated unchanged. It has been reported that in humans cefaclor is apparently not metabolized in the liver [64]. In recent studies it has been postulated that cefaclor is metabolized to a certain extent in the liver and that this biotransformation step is linked to rare cases of serum sickness-like reactions in patients receiving cefaclor [231].

Excretion. Cefaclor is mainly, but not entirely, excreted unchanged into urine. Between 45-80% of a single oral dose has been reported to be excreted within eight hours in adults with normal renal function [218, 227, 232-234]. Urinary concentrations are very high such as 388 μg/ml in urine collected 0-6 h after a 0.5 g oral dose of cefaclor [220]. Renal clearance was reported to be as high as 400 ml/min indicating substantial tubular secretion [227, 232]. This is consistent with the finding that coadministration of probenecid prolonged cefaclor half-life from 0.8 to 1.3 h and decreased the fraction excreted unchanged into urine over the first four hours from 51% to 34% [234, 235]. The remainder of the drug that is not renally eliminated seems to be degraded in serum due to a pH dependent chemical instability [229]. These studies showed that cefaclor is stable at pH <5, but chemically degrades *in vitro* with a half-life of approximately 2 h at physiological pH.

Cefaclor is excreted into the bile only to a very small extent. After oral administration of 1 g cefaclor to cholecystectomized patients provided with a T tube, a mean biliary peak concentration of 7.6 μg/ml was observed at the 3rd hour [236]. Cumulative biliary excretion amounted to only 0.05% of the administered dose.

Pharmacokinetic Parameters. In subjects with normal renal function cefaclor is excreted with a relatively short half-life of 0.5-0.7 h [224, 227, 232, 237]. The oral clearance (CL/F) was reported between 378-411 ml/min, and the apparent oral volume of distribution (Vd/F) of cefaclor is 0.30-0.39 l/kg, equivalent to 21-27 l for a 70 kg subject [224, 238]. Urinary recovery of intact cefaclor is approximately 60% with a range from 45-79% [216, 218, 227, 232, 234].

Dosage Guidelines. The usual adult dose of cefaclor is 250 mg every 8 hours. For severe infections or those caused by less susceptible organisms, 500 mg may be given every 8 hours. The maximum dose is 4g/day.

The usual dose for children 1 month of age or older is 20 mg/kg daily in divided doses every eight hours. For severe infections, otitis media or infections caused by less susceptible organisms, a pediatric dose of 40 mg/kg and a maximum daily dose of 2 g is recommended. Safe use of cefaclor in infants younger than 1 month of age has not been established.

Assay

Several methods for the determination of cefaclor in biological samples by HPLC have been published [229, 239-244]. Cefaclor is usually obtained from plasma by extraction or precipitation with organic solvents. Precipitation with acid is a simpler and more convenient method, taking into account that cefaclor is more stable in acidic solutions [229, 230, 245-247].

Stability

Cefaclor is reportedly unstable in aqueous solution, plasma and microbiological growth media (broth) and even in solid phase [229, 230, 245, 246, 248]. This is of considerable importance when performing assays, for unless specimens are dealt with in an immediate and consistent manner, serious error may occur. When MICs are determined, it is assumed that the concentration of the drug in the growth media stays unchanged for the duration of the experiment. Cefaclor has been reported to be chemically unstable in solution at 37°C [229, 230]. The instability in broth needs to be taken into account when estimating MIC values, to correct for the remaining concentration values at the end of the incubation period.

Antibacterial effect

Cefaclor, like all β-lactam antibiotics, is mainly bactericidal. It inhibits the final stage of bacterial cell wall synthesis by preferentially binding to specific penicillin-binding proteins (PBPs) that are located inside the bacterial cell wall. Penicillin-binding proteins are responsible for several steps in the synthesis in the cell wall and are found in quantities of several hundred to several thousand molecules per bacterial cell. Penicillin-binding proteins vary among different bacterial species. Thus, the intrinsic activity of cefaclor as well as the other cephalosporins and penicillins against a particular organism depends on their ability to gain access to and bind with the necessary PBP. Like all β-lactam antibiotics, cefaclor's ability to interfere with PBP-mediated cell wall synthesis ultimately leads to lysis and bacterial cell death.

Cefaclor is active against gram-positive cocci, including penicillin-resistant strains, as well as against many gram-negative organisms [211, 213].

Since its development, cefaclor has been studied in numerous clinical trials and has been used as a comparative agent in many other studies [249-267].

Reviews about the pharmacokinetic properties [211] and clinical applications and pharmacoeconomics of cefaclor [268]have been published recently.

Examination of the ranges of reported susceptibility data show a substantial amount of variability, but the overall data show that cefaclor is an active agent against these species.

CHAPTER 3
ANALYTICAL METHODS, STABILITY AND PROTEIN BINDING STUDIES

Drug Assay

In order to obtain pharmacokinetic data from plasma and microdialysis samples, a sensitive and selective assay method for the quantitation of cefaclor in human plasma and microdialysis samples was required. The aim of this study was to develop and validate a method for the determination of cefaclor in human serum and microdialysis samples.

Our aim was to reach a lower limit of quantitation than reported, while using a broader range of concentrations and keeping the method as simple as possible. In the previous studies, cefaclor is usually obtained from plasma by extraction or precipitation with organic solvents. We felt precipitation with acid is a simpler and more convenient method, taking into account that cefaclor is more stable in acidic solutions [229, 230, 245-247].

Chemicals and Reagents

For all studies, Cefaclor (CCL) reference standard (Batch # 95010) was provided by ACS DOBFAR, (Hamburg, Germany) and Hexal AG, Germany (Holzkirchen, Germany). The compound was stored in a refrigerator in the original opaque vial. All reagents were of analytical grade and HPLC solvents were of Optima® grade. All chemicals were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). Microbiological assay media and supplies were purchased from Remel, ultrafiltration tubes were Amicon, and microdialysis probes from CMA. Human plasma was obtained from Civitan Regional Blood System, Gainesville, FL. Rat plasma was obtained from Wistar rats (Sprague-Dawley).

Sample Treatment

Plasma samples were thawed at room temperature, vortexed and processed. Plasma samples (400 µL) were precipitated with 160 µL of 12% perchloric acid. The mixture was vortexed for 5 seconds, followed by a centrifugation step of 7 minutes at level 7 (Fisher Centrifuge model 235V). The supernatant was transferred into capped autosampler vials and 50 µL were injected into the HPLC system.

Instrumentation

Pump:	LDC Constametric III
Autosampler:	Perkin Elmer ISS-100
Analytical column:	C18, 5µm reversed phase column (Zorbax ODS, Mac-Mod)
UV-detector:	Spectromonitor D (LDC) variable UV detector
Integrator:	HP 3392A

Chromatographic Conditions

The assay was conducted at room temperature (24-26°C). Conditions of the assay are listed in Table 1.

Table 1: Chromatographic conditions of the cefaclor assay

	Plasma	Microdialysis
Mobile phase:	0.05 M NaAc/Hac:Isopropanol (97%:3%) pH of the aqueous phase: 5.9 degassed under vacuum before use	
Flow rate:	1 mL/min	1.5 mL/min
Injection volume:	50 µL	50 µL
Detection wavelength:	254 nm	254 nm
Retention times:	approx. 9 min	approx. 6 min
Total run times:	20 min	12 min

Quantification

Calibration was carried out by an external standardization method. Calculations were performed using Microsoft Excel®. Least squares linear regression was performed on the calibration curve values using the Linest function. The quality control concentrations and their percent deviation from the theoretical values were calculated from the equations obtained. The run was accepted if not more than 1/3 of the quality controls showed a deviation from the theoretical values equal or greater than 15%.

Assay Validation

The method was validated in a low range and a high range of concentrations for both plasma and microdialysis samples (Table 2). The validation covered linearity, sensitivity, specificity, accuracy and precision, recovery and stability.

Table 2: Concentration ranges studied for both assays

Concentration range	Plasma	Microdialysis
Low	0.5 – 20 µg/mL	0.2 – 3.0 µg/mL
High	1.0 - 200 µg/mL	0.5 – 20 µg/mL

Linearity

Low range. The six calibration curves constructed over the range 0.5 to 20 µg/mL for plasma were linear (r^2>0.999) as well as the six calibration curves constructed over the range 0.2 to 3 µg/mL for microdialysis (r^2>0.992).

Seven cefaclor concentrations of 0, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 µg/mL were used for the standard plasma calibration curve. Seven cefaclor concentrations of 0, 0.2, 0.3, 0.5, 1.0, 2.0 and 3.0 µg/mL were used for the standard microdialysis calibration curve. The precision ranged from 4.9% at 0.5µg/mL to 0.3% at 20.0µg/mL and from

4.1% at 0.2µg/mL to 4.5% at 3.0µg/mL for plasma and microdialysis respectively. The inaccuracy ranged from –5.7% at 0.5µg/mL to –0.3% at 20.0µg/mL and from -3.7% at 0.2µg/mL to 0.2% at 3.0µg/mL for plasma and microdialysis respectively. The inaccuracy was not dependent on the concentration showing that the regression method was suitable for the concentration range used. Typical calibration curves are shown in Figure 12.

High range. The six calibration curves constructed over the range 1.0 to 200µg/mL for plasma were linear (r^2>0.992) as well as the five calibration curves constructed over the range 0.5 to 20µg/mL for microdialysis (r^2>0.982). Eight cefaclor concentrations of 0, 1.0, 5.0, 10.0, 20.0, 50.0, 100.0 and 200.0µg/mL were used for the standard plasma calibration curve. Seven cefaclor concentrations of 0, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0µg/mL were used for the standard microdialysis calibration curve. The precision ranged from 5.8% at 1.0µg/mL to 1.6% at 200.0µg/mL and from 6.0% at 0.5µg/mL to 11.3% at 20.0µg/mL for plasma and microdialysis respectively. The inaccuracy ranged from –0.2% at 1.0µg/mL to 0.4% at 200.0µg/mL and from -2.4% at 0.5µg/mL to 0.5% at 20.0µg/mL for plasma and microdialysis respectively. The inaccuracy was not dependent on the concentration showing that the regression method was suitable for the concentration range used. Typical calibration curves for both plasma and microdialysis are shown in Figure 12.

Both for the low and high ranges, outliers (values with a deviation>20% from the theoretical value) were not taken into account in the calculations.

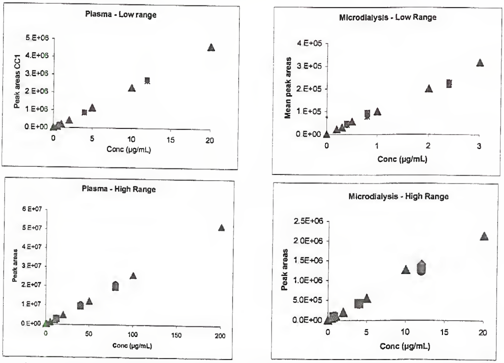


Figure 12: Typical calibration curves for plasma and microdialysis, low- and high-concentration ranges. Symbols represent calibration curve points (▲) and quality control points (✳, ■, ●, ◆)

Sensitivity

Using undiluted samples, the lowest standard concentration used in the calibration curve was 0.5µg/mL for the plasma low-range curve, and 0.2µg/mL for the microdialysis low-range curve. Therefore, the lower quantitation limit was defined as 0.5µg/mL and 0.2µg/mL for plasma and microdialysis samples, respectively. At this concentration the precision value was 4.9% for plasma and 4.1% for microdialysis.

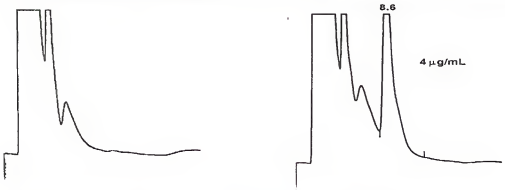
Low range. The intra-and interassay inaccuracy and precision, determined from the lowest quality control at 0.8µg/mL were ≤6.1% for the plasma assay. The same parameters determined from the lowest quality control at 0.4µg/mL were ≤9.5% for the microdialysis assay.

High range. The intra-and interassay inaccuracy and precision, determined from the lowest quality control at 4.0µg/mL were ≤11.5% for the plasma assay. The same parameters determined from the lowest quality control at 0.8µg/mL were ≤12.1% for the microdialysis assay. Both for the low and high ranges, outliers (values with a deviation>20% from the theoretical value) were not taken into account in the calculations.

Specificity

Blank plasma samples from two pooled plasma bags were assayed to determine whether endogenous components were co-eluted at the retention time of cefaclor. No interfering peaks were observed in the background signal of blank plasma samples of two pooled plasma bags. Thus, the assay appears to be specific for cefaclor. Similarly, the blank microdialysis samples were free of interfering peaks.

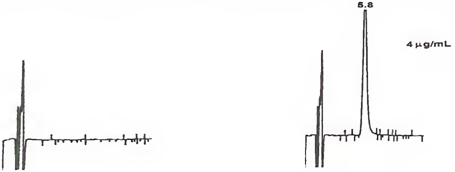
Figure 13 and Figure 14 show examples of typical blank and spiked plasma and microdialysis chromatograms, respectively.



Blank plasma chromatogram Spiked plasma chromatogram

Run time: 20 min; retention time: approx. 9 min; flow rate: 1 mL/min

Figure 13: Typical plasma chromatograms



Blank microdialysis chromatogram Spiked microdialysis chromatogram
Run time: 12 min; retention time: approx. 6 min; flow rate: 1.5 mL/min

Figure 14: Typical microdialysis chromatograms

Stability

The stability of cefaclor was studied in different media and in different conditions. First, the stability was studied in the microdialysis samples matrix (Ringer's solution) in the freezer and the effect of up to 8 freeze-thaw cycles was determined at 2 different concentrations. Then, the stability of both the microdialysis samples and the precipitated plasma samples in the autosampler at room temperature was investigated. Finally, the stability of the drug in various bacterial growth media was determined.

Recovery

Cefaclor was obtained from plasma by precipitating the plasma proteins with 12% perchloric acid, not by solvent extraction, therefore a 100 % recovery is expected [247]. The recovery was assessed by comparing 8 quality controls, 4 prepared in plasma and 4 in distilled water. All samples were treated in the same way. Concentrations were back-calculated using the equation obtained from the linear regression of the plasma samples. The deviation of the aqueous respect of the plasma samples was –11% at 0.5µg/mL to –25% at 10µg/mL. The recovery values expressed as concentrations in µg/mL (Table 3) show we have 100% recovery.

Table 3: Comparative recovery from plasma and aqueous samples

C_{theor} (µg/mL)	Plasma (µg/mL)	Aqueous (µg/mL)	Inaccuracy (%)
0.5	0.55	0.44	11.3%
1.0	1.08	0.95	4.77%
5.0	5.38	4.44	11.3%
10.0	9.80	7.50	25.0%

Accuracy and Precision

The intra-assay accuracy and precision parameters, determined from the analysis of the quality control standards at least five and two times each in one sequence for the low and high range respectively, are shown in Tables Table 4 and Table 5.

Table 4: Intra-assay parameters; low range

	Plasma - low range			Microdialysis - low range		
	QCA 0.80	QCB 4.00	QCC 12.0	QCA 0.40	QCB 0.80	QCC 2.40
Mean	0.80	4.08	12.0	0.40	0.80	2.45
S.D.	0.02	0.11	0.40	0.04	0.02	0.19
CV (%)	2.6%	2.8%	3.3%	9.5%	2.3%	7.8%
N	5	5	5	5	6	6
Min	0.78	3.87	11.3	0.35	0.78	2.31
Max	0.82	4.15	12.4	0.46	0.82	2.81
Inacc (%)	-0.5%	-1.9%	-0.1%	0.2%	-0.5%	-1.9%

Table 5: Intra-assay parameters; high range

	Plasma - high range				Microdialysis - high range		
	QCA 4.0	QCB 12.0	QCC 40.0	QCD 80.0	QCA 0.8	QCB 4.0	QCC 12.0
Mean	4.07	12.5	40.2	82.7	0.79	3.87	12.2
S.D.	0.17	1.0	2.8	3.5	0.10	0.16	0.70
CV (%)	4.2%	8.0%	6.9%	4.2%	12.1%	4.1%	5.7%
N	6	5	6	4	3	2	4
Min	3.86	10.89	34.9	78.8	0.72	3.76	11.1
Max	4.33	13.3	42.8	85.8	0.85	3.98	12.7
Inacc(%)	-1.6%	-4.0%	-0.6%	-3.4%	1.5%	3.3%	-1.4%

The inter-assay accuracy and precision, determined from the analysis of the quality control standards in at least nine and six times each in one sequence for the low and high range respectively are shown in Table 6 and Table 7.

Table 6: Inter-assay parameters; low range

	Plasma - Low range			Microdialysis - Low range		
	QCA 0.80	QCB 4.00	QCC 12.0	QCA 0.40	QCB 0.80	QCC 2.40
Mean	0.77	3.97	11.6	0.39	0.79	2.37
S.D.	0.05	0.29	0.76	0.03	0.03	0.18
CV (%)	6.1%	7.3%	6.6%	8.1%	3.8%	7.7%
N	10	10	10	9	10	10
Min	0.69	3.36	9.7	0.35	0.73	2.14
Max	0.82	4.24	12.4	0.46	0.82	2.81
Inacc (%)	4.1%	0.7%	3.0%	3.5%	1.4%	1.3%

Table 7: Inter-assay parameters; high range

	Plasma - High range				Microdialysis - High range		
	QCA 4.0	QCB 12.0	QCC 40.0	QCD 80.0	QCA 0.8	QCB 4.0	QCC 12.0
Mean	3.85	12.0	39.5	83.0	0.79	3.87	11.8
S.D.	0.44	1.10	2.49	3.69	0.06	0.36	0.81
CV (%)	11.5%	9.2%	6.3%	4.5%	7.8%	9.2%	6.9%
N	10	9	10	8	7	6	8
Min	2.78	10.45	34.9	78.8	0.68	3.57	10.6
Max	4.33	13.3	42.8	88.7	0.88	4.39	12.9
Inacc (%)	3.9%	0.2%	1.4%	-3.7%	1.4%	3.4%	1.8%

Stability Studies

Gillett et al studied the stability of cefaclor in different media, at different temperatures and pH values. Stability was seen to improve with a decrease in temperature and pH. At –20°C no loss of activity was detected in three weeks [230].

An article by Foglesong studies the stability of cefaclor in aqueous solutions and plasma at different temperatures and pH values [229]. The data was used to prepare an Arrhenius plot for Cefaclor at pH 6. The first order degradation constant of cefaclor in aqueous solution at both –20°C and –70°C was calculated from the curve equation. In both cases, k was negligible, indicating that cefaclor can be considered stable in the named conditions. According to the literature, cefaclor is stable when frozen at temperatures of at least –20°C [230]. The cefaclor solutions stored at –20°C and –70°C were therefore assumed to be stable for at least a month. It can also be considered that stability was verified by consistent analytical control assays since the control solutions analyzed during each assay batch were identical to the test solution.

Stability in blood was not assessed because the samples were immediately centrifuged at -4°C after collection, and then frozen at –70°C. Stability in serum at room temperature was also not assessed, given that the samples were at room temperature only enough time to thaw and were processed immediately. In the same way, the plasma standards, calibration curves and quality controls were prepared immediately before each run and then discarded.

Stability in Ringer's Solution in the Freezer at –20 °C

The aim of the experiment was to measure the stability of cefaclor in Ringer's solution at -20°C and to determine if freeze-thaw-cycles affect the stability.

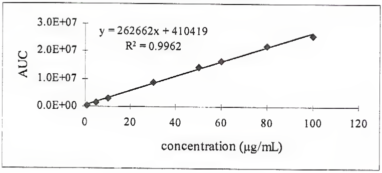
Methods

Cefaclor test solutions (Sol. 1= 100 µg/mL; Sol.2=10 µg/mL) were prepared in Ringer's solution (0.4 g $CaCl_2.2H_2O$, 0.3 g KCl and 9.0 g NaCl / L of distilled water). Ten mL of test solution 1 were kept in the freezer at -20°C for 97 hrs. After 0, 18, 26, 32, 49, 66, 73, 90 and 97 hrs the solution was thawed, a sample was taken and the solution was frozen again. Another 10 mL of test solution 1 were split into 10x1 mL aliquots. The 10 tubes were kept in the freezer at -20°C for 97 hrs. After 0, 18, 26, 32, 49, 66, 73, 90 and 97 hrs every one of the tubes was thawed and a sample was taken. The same two experiments were done with 10 mL of test solution 2. For each experiment samples were taken after 0, 6, 23, 29, 47, 53, 71 and 77 hrs.

Results

The results are shown in Figure 15 to Figure 17:

A: Curve used for the 100 μg/mL samples



B: Curve used for the 10μg/mL samples

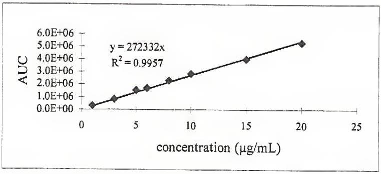
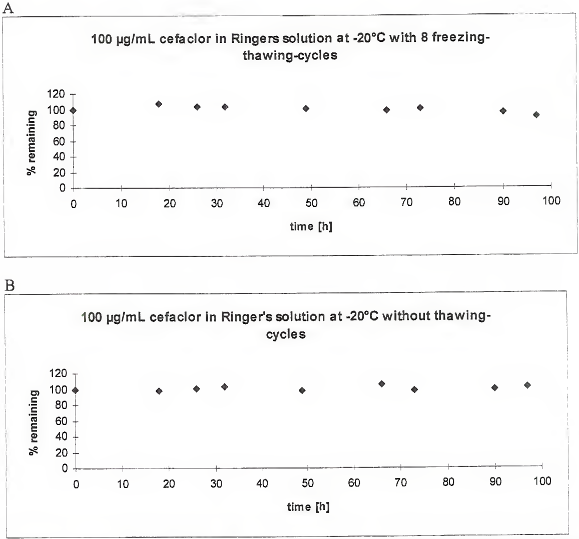


Figure 15: Calibration curves of cefaclor in Ringer's solution.


A

100 µg/mL cefaclor in Ringers solution at -20°C with 8 freezing-thawing-cycles

B

100 µg/mL cefaclor in Ringer's solution at -20°C without thawing-cycles

Figure 16: Stability of a 100 µg/mL solution of cefaclor at –20 °C; with (A) and without (B) up to 8 freeze-thaw cycles.

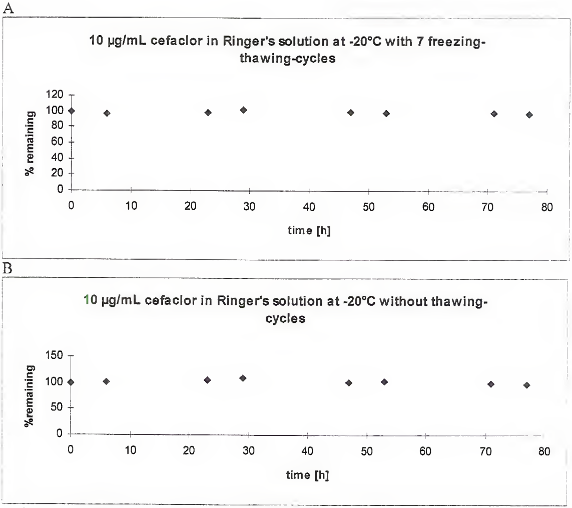


Figure 17: Stability of a 10 µg/mL solution of cefaclor at –20 °C; with (A) and without (B) up to 7 freeze-thaw cycles.

Data analysis and Discussion.

A single factor ANOVA test was used to find out if the degradation of cefaclor in Ringer's solution after 7 to 8 freeze-thaw-cycles is statistically significant ($\alpha=0.05$).

Table 8: Statistical analysis of the results

concentration	treatment	% remaining	P-value
100 µg/mL	after 97 min with 8 freezing-thawing-cycles	90.83%	0.079
	after 97 min without thawing-cycles	102.22%	0.832
10 µg/mL	after 77 min with 7 freezing-thawing-cycles	96.98%	1.000
	after 77 min without thawing-cycles	98.09%	0.566

Cefaclor is stable in Ringer's solution in the freezer at -20°C. Seven to eight freeze-thaw-cycles don't affect the stability. For both of the tested concentrations, 100 µg/mL and 10 µg/mL, the degradation after 7 to 8 freeze-thaw-cycles is statistically not significant (P-value>0.05).

Stability in Ringer's Solution at Room Temperature

The aim of this study was to determine the stability of cefaclor in Ringer's solution at room temperature in the same conditions as during a normal assay run.

Methods

A 1µg/mL solution was prepared and divided into 57 samples. The samples were run automatically for a period of up to 1200 minutes.

Results and Discussion

Figure 18 shows the percent remaining of cefaclor in Ringer's solution at room temperature versus time. This run shows considerable variability. When performing the microdialysis runs, a maximum time period of 300 to 360 minutes for the samples to stay in the autosampler was chosen.

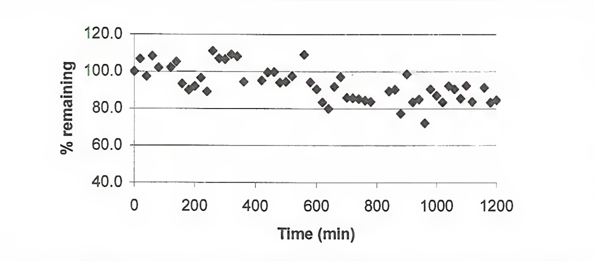


Figure 18: Stability of microdialysis samples in the autosampler. The figure shows the percent remaining of cefaclor versus time of microdialysis samples in the autosampler.

Stability of Precipitated Plasma Samples

The aim of this study was to determine the effect of pH on the stability of cefaclor in the supernatant of precipitated plasma samples during an assay run.

Methods

A 10 µg/mL spiked plasma was prepared and different amounts of sodium acetate were added to the supernatant, to reach the following approximate pH values: 1, 2, 3, 4 and 5. Acidity was measured with pH paper. Each supernatant was divided into 15 samples. The samples were interspersed in the autosampler and assayed up to 1500 minutes.

Results and Discussion

Cefaclor was stable in the supernatant for the entire time period at pH≤3. At higher pH values, degradation was appreciable only after 1200 min. Therefore, it was considered appropriate to conduct the HPLC runs automatically after precipitating with perchloric acid, for at least up to approximately 24 hours.

Figure 19 shows the plot of the percent remaining of cefaclor versus time at pH≤3. The increase in concentrations towards the end of the run may be attributable to sample evaporation. Therefore, capped vials were used in all the sample assays.

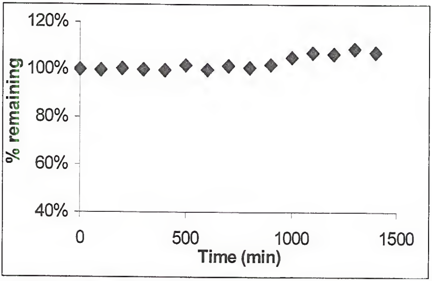


Figure 19: Stability of precipitated plasma samples in the autosampler. The figure shows the percent remaining of cefaclor versus time of precipitated plasma samples at pH≤3.

Stability in Different Bacterial Growth Media

The aim of this study was to measure the stability of cefaclor in three different bacterial growth media: Mueller Hinton Broth, Todd Hewitt Broth and HTM (Haemophilus Test Media) Broth.

Methods

Solutions of cefaclor (100μg/mL) in Mueller Hinton Broth (n=6), Todd Hewitt Broth (n=6) and HTM broth (n=8) were prepared under sterile conditions, divided into 15 x 6.6 mL aliquots and incubated at 37°C. Samples were taken up to a maximum of 24 hours.

The samples were analyzed by isocratic, reversed phase HPLC with UV detection at μ=254 nm with a limit of detection of approximately 1.0 μg/ml. A C18 (Zorbax®

ODS, 5µm, 4.6x150mm) column was used. The method used to measure the broth samples is the same method used for the measurement of plasma samples (0). The method was not validated for broth samples and quality controls were not employed, because the cefaclor concentrations were not backcalculated. Results are expressed as % remaining of the initial peak area versus time. The injection volume was 50 µL and the retention time was approximately 8 minutes at a flow rate of 1 mL/min.

Results and Discussion

The plots of % remaining of cefaclor vs. time for the bacterial growth media studied are shown in Figure 20.

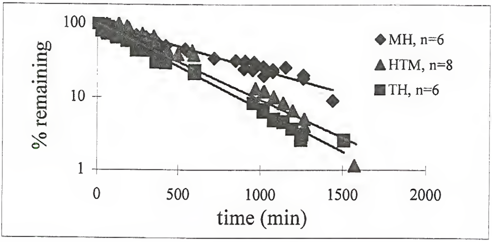


Figure 20: Percent remaining of cefaclor vs. time at 37°C for three different bacterial growth media. MH = Mueller Hinton, HTM = Haemophilus Test Media, TH = Todd Hewitt

The results are summarized below (Table 9). Mean $t_{1/2}$ was calculated averaging the $t_{1/2}$ values obtained from individual runs. Standard deviation was calculated using the $t_{1/2}$ values obtained from individual runs.

Table 9: $t_{1/2}$ of cefaclor in different growth media at 37°C, normal MIC incubation periods and respective % remaining of the drug after the incubation.

Bacterial growth media	$t_{1/2}$ (minutes) Mean ± S.D.	MIC incubation periods (hours)	% cefaclor remaining after the incubation period
Mueller-Hinton	419±79	20	13.7±5.0
HTM	307±64	24	4.0±2.8
Todd Hewitt	261±22	24	2.2±0.7

Cefaclor is unstable in bacterial growth media. The instability of cefaclor in different growth media directly affects the determination of MICs. This results in an overestimation of the minimum inhibitory concentrations. Thus, the percent of cefaclor remaining after the incubation period in the respective media should be taken into account when determining MICs.

Approximation of $t_{1/2}$ to Physiological Conditions.

In a separate experiment (n = 2), broth solutions were diluted after taking each sample in order to simulate physiological conditions ($t_{1/2}$ approximately 60 minutes). This experiment was done with 10 mL of a 100 µg/mL solution of cefaclor in Todd Hewitt Broth. To obtain a half-life of 1 hour instead of 5 hours the solution had to be diluted after taking each sample. The initial concentration in the flask can be expressed as:

$$C_0 = \frac{D}{V}$$

Equation 16

where V is the total volume in mL and D is the initial amount of drug added to the flask. The concentration C_1 after performing the first dilution at time = t minutes can be expressed as:

$$C_1 = C_0 \cdot e^{-k_e \cdot t}$$

Equation 17

where k_e is the simulated elimination rate constant and t is the time. Considering the dilution method withdraws a certain amount of drug every 15 minutes, C_1 can also be expressed by:

$$C_1 = \frac{D-d}{V} = \frac{D}{V} - \frac{d}{V}$$

Equation 18

where d is the amount of drug withdrawn, and D and V have been defined above. Equation 17 and Equation 18 are equal, therefore:

$$C_0 \cdot e^{-k_e \cdot t} = \frac{D}{V} - \frac{d}{V}$$

Equation 19

substituting the expression for C_0 in Equation 19 and rearranging we obtain:

$$D \cdot (1 - e^{-k_e \cdot t}) = d$$

Equation 20

Substituting D for $D = C_0 \cdot V$ (Equation 16) and d for an equivalent expression ($d = C_0 \cdot v$) and simplifying we obtain:

$$v = V \cdot (1 - e^{-k_e \cdot t})$$

Equation 21

where v is the volume in mL that is necessary to withdraw and replace from the total volume V every t hours to obtain a $t_{½}$ corresponding to $t_{1/2} = \ln 2/k_e$. In the case of cefaclor, its own instability in broth needs to be taken into account and integrated into the formula. In Todd Hewitt broth, the degradation $t_{½}$ of cefaclor at 37°C is approximately 4.35 hours (Table 9), which corresponds to a degradation rate constant of 0.159 h^{-1}. To simulate a $t_{½}$ of approximately 1 hour, the k_e to be simulated (0.534 h^{-1}) will be the difference between the desired k_e (0.693 h^{-1}) and the chemical degradation constant

(0.159 h^{-1}). At 15 minute intervals (t = 0.25 hours) and using a total volume V=10 mL, a volume of approximately 1.25 mL will need to be withdrawn.

Every 15 minutes 1.3 mL of the solution were taken out and 1.3 mL of broth were added. This resulted in a half-life of 59 minutes.

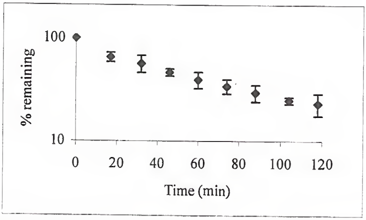


Figure 21: Approximation of the $t_{1/2}$ of cefaclor to 1 hour by stepwise dilution in Todd Hewitt broth at 37°C (n = 2).

Protein Binding Studies

The aim of these studies was to measure the binding of cefaclor to plasma proteins in animal plasma and human plasma.

Methods

Five-mL aliquots of 20, 60 and 100 μg/mL cefaclor solutions in human and in rat plasma were prepared. For each concentration 3 microcentrifuge tubes were prepared with 0.5 mL of solution, this was directly mixed with 200 μL of 12 % perchloric acid and centrifuged for 7 min at approximately 13100 rpm (RCF: 15184 xg). The supernatant was injected. Three other microcentrifuge tubes were prepared in the same way, but before the perchloric acid treatment they were centrifuged at 4°C and 1500xg for 30 min. Three samples of each concentration were ultrafiltrated in 0.5 mL ultrafiltration tubes at 4°C and 1500xg for 30 min. The ultrafiltrate was directly injected. The injection volume was 50 µL. Calibration curves were prepared both in ultrafiltrate and in Ringer's solution. The ultrafiltrate for calibration was obtained by centrifugation as described above, and was acidified to a pH of 6 with acetic acid.

The concentrations of cefaclor in the ultrafiltrate were compared to the total concentrations in plasma. The samples were analyzed by isocratic, reversed phase HPLC with UV detection at µ=254 nm with a limit of quantitation of approximately 1.0 µg/ml. A C18 (Zorbax® ODS, 5µm, 4.6x150mm) column was used.

Results

Table 10: Protein Binding of Cefaclor in Human Plasma

protein binding in %				
ultrafiltrate conc. calculated with calibration curve in Ringer's solution	Nr.	20 µg/mL	60 µg/mL	100 µg/mL
	1	23.7%	25.7%	33.1%
	2	24.4%	42.0%	46.3%
	3	27.8%	9.9%	11.2%
	4	10.9%	7.2%	4.2%
	mean	21.7%	21.2%	23.7%
ultrafiltrate conc. calculated with calibration curve in ultrafiltrate	1	9.8%	16.7%	26.0%
	2*)			
	3	25.0%	6.4%	7.7%
	4	17.0%	13.6%	10.8%
	mean	17.3%	12.2%	14.8%

*) Nr. 2 could not be calculated with the ultrafiltrate calibration curve because a degradation of cefaclor occurred in the calibration solutions.

Table 11: Protein Binding of Cefaclor in Rat Plasma

protein binding in %				
	Nr.	20 µg/mL	60 µg/mL	100 µg/mL
ultrafiltrate conc. calculated with calibration curve in Ringer's solution	1	31.2%	26.8%	25.1%
	2	31.0%	20.5%	13.2%
	3	39.1%	36.1%	39.9%
	4	20.5%	18.1%	13.8%
	5	35.8%		
	6	24.7%		
	avg.	30.4%	25.4%	23.0%
ultrafiltrate conc. calculated with calibration curve in ultrafiltrate	1	44.4%	40.8%	39.5%
	2	22.3%	9.5%	1.2%
	3	37.2%	34.1%	38.0%
	4	17.8%	15.3%	10.9%
	5	35.6%		
	6	24.6%		
	avg	30.3%	24.9%	22.4%

Nr. 1, 3 and 5 were prepared with fresh rat plasma. Nr. 2, 4 and 6 were prepared in frozen plasma. Because the results in frozen and in fresh plasma are different, it is important to calculate the averages separately:

	fresh plasma		frozen plasma	
conc.[µg/mL]	calc. with Ringer's	calc. with ultrafiltrate	calc. with Ringer's	calc. with ultrafiltrate
20	35.4%	39.1%	25.4%	21.6%
60	31.5%	37.5%	19.3%	12.4%
100	32.5%	38.8%	13.5%	6.1%

Discussion

In human frozen plasma a protein binding of 18.5 +/- 4.4%(range: 12.2 to 23.7%) was found, in rat plasma a protein binding of 16.3 +/-7.0% (range: 6.1 to 25.4%) and 35.8 +/- 3.2% (range: 31.5 to 39.1%) was found in frozen and fresh plasma respectively.

The experiments in rat plasma show that the protein binding in fresh plasma is higher than in frozen plasma. In thawed frozen plasma proteins were precipitated. These proteins are not available to bind the drug and may explain the observed difference.

The experiments in human plasma, which were only done with frozen plasma showed less protein binding than the literature reported, which is approximately 20-25% [22, 64, 269].

CHAPTER 4
ANIMAL PHARMACOKINETIC STUDIES

Aim of the Animal Pharmacokinetic Studies

The aim of this study was to evaluate the pharmacokinetic profile of cefaclor in plasma as well as in muscle and lung in male Wistar rats.

Materials and Methods

Experimental Design

Body weight of the animals was recorded at study initiation. Rats received I.V. (I.V.) doses of either 50 mg/kg or 75 mg/kg of cefaclor. Plasma concentrations as well as unbound tissue concentrations in thigh muscle and lung were measured. Unbound concentrations in thigh muscle and lung were measured by microdialysis. Probe recovery was determined by retrodialysis and allowed conversion of the measured dialysate concentrations to the actual unbound tissue concentrations. Animals were sacrificed at the end of the study.

The study was performed under GLP conditions and with the approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Florida. The animals were housed in the Animal Resources Department of the University of Florida and kept according to the Animal Resources standard husbandry procedures.

The data were analyzed both by non-compartmental and compartmental pharmacokinetic approaches.

Dose Administration

Fourteen male Wistar rats were used for this experiment (Breeder: Harlan Sprague Dawley, Inc.). Their body weights were approximately 300-400 g (animals with similar body weights were used), corresponding to approximately 2 months of age. The animals were numbered in the sequence of the experiments. The letter A designates the first dose group (50 mg/Kg) and the letter B the second dose group (75 mg/Kg). No identifying devices such as tattoos or collar numbers were used given that the surgeries were terminal and performed one at a time.

Seven of the 14 male animals received 50 mg/Kg of cefaclor IV and the other seven received 75 mg/Kg of cefaclor IV.

The test substance was injected as an IV bolus injection over a one-minute period in the femoral vein of the right hind leg.

Anesthetic Procedure

The animals were initially anesthetized by inhalation of Methoxyflurane gas (Metofane®) in a glass jar. Then a dose of 1.25 g/Kg of ethylcarbamate was administered intraperitoneally. Surgical anesthesia resulted after about 4-5 minutes, and was confirmed by the absence of reflexes after pinching the footpads.

Subsequent doses of ethylcarbamate (equal to one half the first dose) were administered to the animal whenever consciousness signs were observed.

Animals were immobilized in a supine position; body temperature was kept at 37°C with a lamp.

Blood Samples

For all blood sampling a small incision was made in the neck area and a catheter (Venflon®, 20-22 gauge) was inserted in the carotid artery at the beginning of the experiment. The artery was irrigated after each sampling with an equal volume of heparinized saline (25 UI of sodium heparin/mL of normal saline).

Blood samples (400-500 µL) were collected in heparinized tubes (sodium heparin), before cefaclor administration (time 0) and at times 5, 10, 15, 30, 45, 60, 90 and 120 minutes after administration. The blood samples were centrifuged 3 minutes at room temperature at approximately 9170 rpm (RCF: 10629 xg) and the plasma kept at -70 °C until analyzed.

Approximately 400-500 µL of blood/animal/sampling time were obtained, a sufficient volume to obtain approximately 250 µL of plasma/animal/sampling time for posterior determination of the cefaclor levels by HPLC.

Microdialysis Samples

Microdialysis probes were implanted both in muscle and lung tissue. Microdialysis samples were collected simultaneously for lung and muscle, over 20 minute intervals at times 20, 40, 60, 80, 100 and 120 minutes after administration of the IV cefaclor dose.

Probe Calibration

The microdialysis probes were CMA/10 custom made microdialysis probes, shaft length 70mm, membrane length 16mm, 20kD cutoff.

Both probes were initially perfused with Ringer's (NaCl 0.9%, KCl 0.03%, CaCl2 0.03%) at a flow rate of 3 µL/min using a Harvard Apparatus 22 injection pump, model 55-4150. The probes were allowed to equilibrate for 20 minutes after insertion.

After equilibration, the probes were calibrated by retrodialysis. The syringes were changed and a solution of 10 mg/mL of cefaclor was perfused through the probes for 40 minutes at a flow rate of 3 μL/min.

Samples were collected during the following periods: 10-30 minutes (after the beginning of retrodialysis), and 30-50 minutes. The first 10 minutes of the retrodialysis infusion were not collected, to account for the dead space in the tubing.

After the retrodialysis samples were obtained, the syringes were changed back and the probes were again perfused with Ringer's solution at a flow rate of 3 μL/min for 20 minutes to allow for washout of the retrodialysis solution. The solution used for the retrodialysis was kept frozen at –70°C until analyzed.

After washout was complete, the IV cefaclor dose was administered and the time count began again.

The calibration for the muscle and lung probes was done in each animal before the experiment was performed.

Muscle Microdialysis

The left hind leg muscle was used for insertion of a microdialysis probe after skin removal. The probe was introduced into the muscle through the tip of a 20-gauge needle that was removed after placing the probe.

Lung Microdialysis

The rats were intubated through a tracheotomy and artificially ventilated with room air throughout the experiment using a rodent respirator (Harvard Apparatus; model 683), with a frequency of 62-66 min^{-1} and a volume of 2 mL.

The right lung was exposed through partial resection of the ribs. A microdialysis probe was inserted into the intermediate lobe of the right lung through a small incision made in the pleura. The probe was held in place with ties around the probe shaft and the lung and then the lobe was carefully put back in place.

Individual Non-compartmental Pharmacokinetic Analysis

The following parameters were calculated for each animal:

Plasma.

The terminal elimination rate constant (k_e) was calculated by linear regression of the natural logarithms of the last n plasma concentrations. Terminal half-life was calculated as $\ln(2)/k_e$. The initial concentration C_0 was determined by logarithmic back-extrapolation to t=0 using the first two data points. The area under the curve (AUC) was calculated using the trapezoidal rule up to the last data point (C_x) and adding the extrapolated terminal area, calculated as C_x/k_e. The area under the first moment curve (AUMC) was calculated from a plot of C·t vs. t using the trapezoidal rule up to the last data point (C_x) at time tx and adding the extrapolated terminal area, calculated as $C_x \cdot t_x/k_e + C_x/k_e^2$. The mean residence time (MRT) was calculated as AUMC/AUC. The volume of distribution of the central compartment (Vc) was calculated as D/C_0, where D is the dose. The volume of distribution at steady state (Vdss) was calculated as $D \cdot AUMC/AUC^2$. The clearance (CL) was calculated as D/AUC.

Muscle and Lung.

Unbound concentrations in muscle and lung were calculated from the measured microdialysate concentrations and the measured recovery from retrodialysis samples. The terminal elimination rate constant (k_e) was calculated by linear regression of the natural logarithms of the last n plasma concentrations. Terminal half-life was calculated as

$\ln(2)/k_e$. The highest concentration (C_{max}) was obtained directly from the experimental data, together with the respective time of maximum concentration (t_{max}). The area under the curve (AUC) was calculated using the trapezoidal rule up to the last data point (C_x) and adding the extrapolated terminal area, calculated as C_x/k_e. The area under the first moment curve (AUMC) was calculated from a plot of C·t vs. t using the trapezoidal rule up to the last data point (C_x) at time t_x and adding the extrapolated terminal area, calculated as $C_x \cdot t_x/k_e + C_x/k_e^2$. The mean residence time (MRT) was calculated as AUMC/AUC. The tissue distribution (or tissue penetration) factor F was calculated as the ratio of the unbound AUC in plasma and the unbound AUC in tissue ($AUC_T/f_u \cdot AUC$), where fu is the fraction unbound in plasma.

Compartmental Pharmacokinetic Analysis

Plasma.

For the compartmental pharmacokinetic data analysis of the plasma concentrations, a two-compartment body model without absorption was used. The respective equation for the plasma concentration C is

$$C = a \cdot e^{-\alpha \cdot t} + b \cdot e^{-\beta \cdot t}$$

Equation 22

where α and β are the hybrid constants representing distribution and elimination, a and b are the respective intercepts, and t is time.

Muscle and Lung.

Unbound muscle and lung concentrations (C_T) were fitted simultaneously with the respective plasma concentrations. The respective equation for the unbound concentration in the tissue is:

$$C_T = \frac{F \cdot f_u \cdot (a \cdot \beta + b \cdot \alpha)}{(\beta - \alpha)} \cdot \left(e^{-\alpha \cdot t} - e^{-\beta \cdot t}\right)$$

Equation 23

where α, β, a and b have been defined above, f_u is the fraction unbound in plasma and F is a proportionality factor characterizing the degree of tissue distribution.

The data was analyzed by nonlinear regression using the program Scientist (MicroMath, Salt Lake City, Utah). For each animal, the plasma concentrations were fitted to both models. The coefficient of determination (CD) as well as the Model Selection Criterion (MSC) were used as a criterion for the goodness of the resulting curve fits. The closer CD is to 1, the better the agreement between measured and calculated values. The higher MSC, the more appropriate the selected model.

Results

Recovery

The results for the recovery for all animals (n=14) calculated by retrodialysis are listed in Table 12. The mean recovery was calculated using the means of the perfusate and dialysate measurements for all animals. The standard deviation (S.D.) was calculated from the individual recoveries. The coefficient of variation (C.V.) was calculated as the S.D./mean.

Table 12: Recovery

	Muscle	Lung
Mean	39%	33%
S.D.	12%	7%
C.V.	30%	22%

Comparison of Plasma, Muscle and Lung Pharmacokinetics after I.V. Administration of 50 mg/kg Cefaclor

Individual Non-compartmental Pharmacokinetic Analysis

Plasma. The results of the individual non-compartmental pharmacokinetic data analysis for cefaclor after I.V. administration of 50 mg/kg for the plasma data are listed in Table 13. The initial concentration was 248 µg/ml, which declined with a median terminal half-life of 56 minutes. The median area under the curve was 7992 µg/ml·min, equivalent to a total body clearance of 6.3 ml/min/kg. The mean residence time was 69 minutes. The volume of distribution of the central compartment was 0.20 L/kg, and Vdss and Vdarea were found to be 0.55 and 0.60 L/kg, respectively. The median unbound area under the curve in plasma (AUC_f), based on a protein binding of 36%, was 5115 µg/ml·min.

Muscle. The results of the individual non-compartmental pharmacokinetic data analysis for cefaclor after I.V. administration of 50 mg/kg are listed in Table 14. The results are shown for the unbound muscle concentrations, based on an average recovery of 33±7%. The recoveries used for both muscle and lung are the means of the recoveries for each tissue for 14 animals, calculated as the average percent deviation of the retrodialysis samples from the retrodialysis solution.

The unbound muscle concentrations declined with a median terminal half-life of 53 minutes. The median area under the curve was 1341 µg/ml·min, resulting in a median tissue distribution factor of 0.26. The maximum concentration was 28.2 µg/ml after 10 minutes. The mean residence time was 69 minutes.

Lung. The results of the individual non-compartmental pharmacokinetic data analysis for cefaclor after I.V. administration of 50 mg/kg are listed in Table 15. The results are shown for the unbound lung concentrations, based on an average recovery of 39±12%.

The unbound lung concentrations declined with a median terminal half-life of 56 minutes. The median area under the curve was 1108 µg/ml·min, resulting in a median tissue distribution factor of 0.23. The maximum concentration was 27.4 µg/ml after 10 minutes. The mean residence time was 71 minutes.

Compartmental Pharmacokinetic Analysis

A two-compartment model without absorption was able to produce a good curve fit of the average plasma concentrations (Figure 22). The coefficient of determination (r^2) was 0.978, the model selection criterion (MSC) was 3.2, indicating reasonable curve fits.

The respective parameters were a=786±2170 µg/ml, b=101±13 µg/ml, α=0.44±0.54 min^{-1}, β=0.016±0.002 min^{-1} and F=0.23±0.08.

As has been observed in many other studies before, the first data point of both muscle and lung concentration is not well described by the model since the distribution equilibrium is not established, yet. However, in the terminal phase of the curve, the model describes both plasma and unbound tissue concentrations very well.

Table 13: Results of the individual non-compartmental pharmacokinetic analysis (50 mg/kg I.V.)
Plasma

Time (min) \ Rat	**1A'**	**2A'**	**3A'**	**4A'**	**6A'**	**7A'**	**8A'**	**Mean**	**S.D.**	**Median**
5	131.5	171.8	165.5	132.0	199.8	249.7	169.5	174.3	40.9	169.5
10	78.9	123.6	102.1	79.6	130.9	158.5	116.0	112.8	28.6	116.0
15	70.4	94.8	73.8	59.6	99.6	112.3	84.9	85.1	18.4	84.9
30	46.5	58.6	47.1	31.7	57.4	74.8	66.1	54.6	14.2	57.4
45	34.5	45.1	28.6	24.0	41.4	78.9	53.0	43.6	18.4	41.4
60	28.2	37.0	24.8	19.1	37.5	59.1	51.5	36.7	14.4	37.0
90	18.4	25.3	12.9	12.8	30.6	53.5	40.1	27.7	15.0	25.3
120	15.2	19.0	8.9	9.3	31.7	51.7	37.4	24.8	16.1	19.0
ke [min^{-1}]	0.012	0.012	0.018	0.013	0.006	0.005	0.006	0.010	0.005	0.012
n	5	5	5	5	5	5	5	5	0	5
$t_{1/2}$ [min]	55.8	56.0	38.2	51.6	113.8	144.7	113.0	81.9	41.0	56.0
C_0 [μg/ml]	219.2	239.0	268.3	218.9	305.1	393.4	247.6	270.2	62.1	247.6
AUC[μg/ml·min]	6160	7992	5447	4686	12343	20961	13699	10184	5871	7992
AUMC[μg/ml·min^2]	421627	540871	228774	257723	1749084	3982033	2056753	1319552	1387243	540871
MRT[min]	68.4	67.7	42.0	55.0	141.7	190.0	150.1	102.1	57.4	68.4
Vc [ml/g]	0.23	0.21	0.19	0.23	0.16	0.13	0.20	0.19	0.04	0.20
Vdss [ml/g]	0.56	0.42	0.39	0.59	0.57	0.45	0.55	0.50	0.08	0.55
Vdarea [ml/g]	0.65	0.51	0.51	0.79	0.66	0.50	0.60	0.60	0.11	0.60
CL [ml/min/kg]	8.1	6.3	9.2	10.7	4.1	2.4	3.6	6.3	3.1	6.3
AUC_f[μg/ml*min]	3943	5115	3486	2999	7899	13415	8767	6518	3757	5115

Table 14: Results of the individual non-compartmental pharmacokinetic analysis (50 mg/kg I.V.)
Unbound Concentrations in Muscle

Time (min) \ Rat	**1A'**	**2A'**	**3A'**	**4A'**	**6A'**	**7A'**	**8A'**	**Mean**	**S.D.**	**Median**
10	32.1	23.9	28.2	22.4	22.1	31.7	35.7	28.0	5.4	28.2
30	15.1	13.6	11.9	9.8	12.2	17.7	16.5	13.9	2.8	13.6
50	9.3	8.2	6.1	4.9	7.1	11.6	10.5	8.3	2.4	8.2
70	6.4	6.5	3.9	3.5	5.3	9.1	8.1	6.1	2.0	6.4
90	4.5	4.8	2.7	2.7	4.7	8.3	7.0	5.0	2.1	4.7
110	4.3	3.7	1.9	2.0	3.7	7.2	6.4	4.2	2.0	3.7
ke [min^{-1}]	0.013	0.013	0.019	0.015	0.010	0.008	0.008	0.012	0.004	0.013
n	4	4	4	4	4	4	4	4		4
$t_{1/2}$ [min]	52.5	52.2	36.3	47.7	67.5	91.0	84.8	61.7	20.2	52.5
C_{max} [µg/ml]	32.1	23.9	28.2	22.4	22.1	31.7	35.7	28.0	5.4	28.2
t_{max} [min]	10	10	10	10	10	10	10	10.0	0.0	10.0
AUC[µg/ml·min]	1561	1341	1038	915	1318	2432	2222	1547	576	1341
AUMC[µg/ml·min^2]	104783	92953	45358	50142	111946	290365	236334	133126	93836	104783
MRT[min]	67.1	69.3	43.7	54.8	84.9	119.4	106.4	77.9	27.3	69.3
F	0.40	0.26	0.30	0.30	0.17	0.18	0.25	0.27	0.08	0.26

Table 15: Results of the individual non-compartmental pharmacokinetic analysis (50 mg/kg I.V.)
Unbound Concentrations in Lung

Time (min) \ Rat	**1A'**	**2A'**	**3A'**	**4A'**	**6A'**	**7A'**	**8A'**	**Mean**	**S.D.**	**Median**
10	32.5	19.1	27.4	24.4	36.6	46.9	17.4	29.2	10.4	27.4
30	16.2	11.7	10.8	7.2	15.0	20.6	6.7	12.6	5.0	11.7
50	9.5	5.8	5.9	4.2	10.2	14.5	4.5	7.8	3.8	5.9
70	6.6	4.6	3.6	3.2	7.4	12.6	3.7	5.9	3.3	4.6
90	5.4	3.7	2.6	2.5	5.9	10.3	3.2	4.8	2.8	3.7
110	4.3	2.9	1.8	1.8	4.8	9.1	3.0	4.0	2.5	3.0
ke [min^{-1}]	0.013	0.011	0.019	0.014	0.012	0.008	0.007	0.012	0.004	0.012
n	4	4	4	4	4	4	4	4		4
$t_{1/2}$ [min]	53.8	61.0	36.6	48.9	56.4	86.5	105.0	64.0	23.6	56.4
C_{max} [µg/ml]	32.5	19.1	27.4	24.4	36.6	46.9	17.4	29.2	10.4	27.4
t_{max} [min]	10	10	10	10	10	10	10	10.0	0.0	10.0
AUC[µg/ml·min]	1618	1089	985	849	1758	3088	1108	1499	776	1108
AUMC[µg/ml·min^2]	108947	82175	42882	44406	124285	344060	143603	127194	102981	108947
MRT[min]	67.3	75.5	43.5	52.3	70.7	111.4	129.6	78.6	31.1	70.7
F	0.41	0.21	0.28	0.28	0.22	0.23	0.13	0.25	0.09	0.23

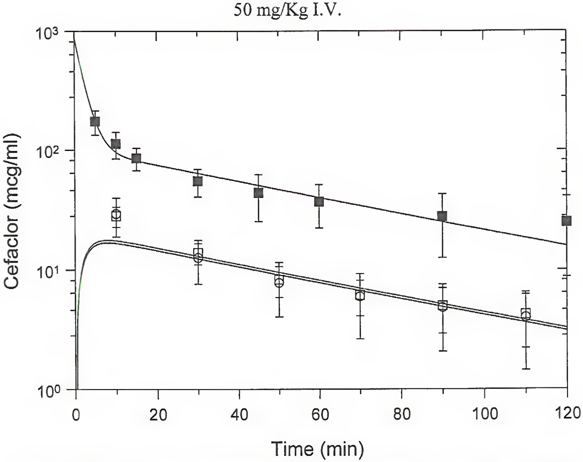


Figure 22: Mean plasma concentrations (± S.D.) of cefaclor (■) and mean unbound concentrations (± S.D.) in muscle (□) and lung (○). The lines represent the respective curve fits from nonlinear regression using simultaneous compartmental pharmacokinetics. (n=7)

Comparison of Plasma, Muscle and Lung Pharmacokinetics after I.V. Administration of 75 mg/kg Cefaclor

Individual Non-compartmental Pharmacokinetic Analysis

Plasma. The results of the individual non-compartmental pharmacokinetic data analysis for cefaclor after I.V. administration of 75 mg/kg for the plasma data are listed in Table 16.

The initial concentration was 216 µg/ml which declined with a median terminal half-life of 59 minutes. The median area under the curve was 5610 µg/ml·min, equivalent to a total body clearance of 13.4 ml/min/kg. The mean residence time was 68 minutes. The volume of distribution of the central compartment was 0.35 L/kg, and Vdss and Vdarea were found to be 0.84 and 0.98 L/kg, respectively. The median unbound area under the curve in plasma (AUC_f), based on a protein binding of 36%, was 3590 µg/ml·min.

Muscle. The results of the individual non-compartmental pharmacokinetic data analysis for cefaclor after I.V. administration of 75 mg/kg are listed in Table 17.

The results shown are for the unbound concentrations in muscle, based on an average recovery of 33±7%.

The unbound muscle concentrations declined with a median terminal half-life of 45 minutes. The median area under the curve was 1011 µg/ml·min, resulting in a median tissue distribution factor of 0.23. The maximum concentration was 20.6 µg/ml after 10 minutes. The mean residence time was 59 minutes.

Lung. The results of the individual non-compartmental pharmacokinetic data analysis for cefaclor after I.V. administration of 75 mg/kg are listed in Table 18. The results shown are for the unbound concentrations in lung, based on an average recovery of 39±12%.

The unbound lung concentrations declined with a median terminal half-life of 54 minutes. The median area under the curve was 1277 μg/ml·min, resulting in a median tissue distribution factor of 0.29. The maximum concentration was 23.5 μg/ml after 10 minutes. The mean residence time was 60 minutes.

Compartmental Pharmacokinetic Analysis

A two-compartment model without absorption was able to produce a good curve fit of the average plasma concentrations (Figure 23). The coefficient of determination (r^2) was 0.977, the model selection criterion (MSC) was 3.2, indicating reasonable curve fits.

The respective parameters were a=596±1655 μg/ml, b=90±12 μg/ml, α=0.43±0.54 min-1, β=0.019±0.002 min-1 and F=0.24±0.08.

As has been observed in many other studies before, the first data point of both muscle and lung concentration is not well described by the model since the distribution equilibrium is not established, yet. However, in the terminal phase of the curve, the model describes both plasma and unbound tissue concentrations very well.

Table 16: Results of the individual non-compartmental pharmacokinetic analysis (75 mg/kg I.V.)
Plasma

Time (min) \ Rat	**10B**	**11B**	**12B**	**13B**	**14B**	**15B**	**16B**	**Mean**	**S.D.**	**Median**
5	203.7	129.4	132.2	123.3	201.9	152.3	85.4	146.9	43.1	132.2
10	137.3	91.7	80.9	88.3	127.3	101.7	57.4	97.8	27.3	91.7
15	85.6	113.0	56.4	59.7	87.6	85.1	45.3	76.1	23.3	85.1
30	52.3	54.5	37.3	33.6	55.7	49.7	27.8	44.4	11.3	49.7
45	39.7	41.6	29.3	26.3	37.4	36.2	15.8	32.3	9.1	36.2
60	27.8	32.8	20.9	16.4	31.9	22.4	14.4	23.8	7.3	22.4
90	19.1	24.1	18.2	11.6	24.2	14.2	10.2	17.4	5.6	18.2
120	19.9	22.3	11.6	9.0	27.8	9.9	8.6	15.6	7.7	11.6
ke [min^{-1}]	0.011	0.010	0.012	0.015	0.007	0.018	0.012	0.012	0.003	0.012
n	5	5	5	5	5	5	5	5	0	5
$t_{1/2}$ [min]	62.3	70.2	56.8	47.0	94.3	38.6	58.9	61.1	17.9	58.9
C_0 [µg/ml]	302.2	182.7	216.1	172.2	320.0	228.0	126.9	221.2	69.7	216.1
AUC[µg/ml·min]	7986	8192	5305	4447	10364	5610	3628	6504	2404	5610
AUMC[µg/ml·min^2]	573856	726232	340301	232425	1199858	250446	246326	509921	358114	340301
MRT[min]	71.9	88.6	64.1	52.3	115.8	44.6	67.9	72.2	23.8	67.9
Vc [ml/g]	0.25	0.41	0.35	0.44	0.23	0.33	0.56	0.37	0.11	0.35
Vdss [ml/g]	0.67	0.81	0.91	0.88	0.84	0.60	1.33	0.86	0.23	0.84
Vdarea [ml/g]	0.84	0.93	1.16	1.14	0.98	0.74	1.66	1.07	0.30	0.98
CL [ml/min/kg]	9.4	9.2	14.1	16.9	7.2	13.4	19.6	12.8	4.5	13.4
AUCf[µg/ml*min]	5111	5243	3395	2846	6633	3590	2322	4163	1539	3590

Table 17: Results of the individual non-compartmental pharmacokinetic analysis (75 mg/kg I.V.)
Unbound Concentrations in Muscle

Time (min) \ Rat	**10B**	**11B**	**12B**	**13B**	**14B**	**15B**	**16B**	**Mean**	**S.D.**	**Median**
10	20.2	20.6	17.6	23.6	25.9	34.0	15.3	22.5	6.2	20.6
30	12.5	11.0	6.7	8.3	14.0	19.8	4.6	11.0	5.1	11.0
50	7.0	6.7	3.9	3.4	7.4	9.3	2.8	5.8	2.4	6.7
70	4.7	5.0	2.8	2.0	5.6	5.6	1.9	3.9	1.7	4.7
90	3.2	3.5	2.5	1.4	4.3	3.5	1.4	2.8	1.1	3.2
110	2.4	2.7	1.9	1.1	3.7	2.6	1.3	2.2	0.9	2.4
ke [min^{-1}]	0.018	0.015	0.011	0.019	0.012	0.021	0.013	0.016	0.004	0.015
n	4	4	4	4	4	4	4	4		4
$t_{1/2}$ [min]	38.4	45.0	61.9	35.9	58.9	32.3	53.1	46.5	11.6	45.0
C_{max} [μg/ml]	20.2	20.6	17.6	23.6	25.9	34.0	15.3	22.5	6.2	20.6
t_{max} [min]	10	10	10	10	10	10	10	10.0	0.0	10.0
AUC[μg/ml·min]	1011	1038	774	723	1362	1419	557	983	324	1011
AUMC[μg/ml·min^2]	53802	62589	54644	26281	98992	60587	32512	55630	23641	54644
MRT[min]	53.2	60.3	70.6	36.4	72.7	42.7	58.4	56.3	13.4	58.4
F	0.20	0.20	0.23	0.25	0.21	0.40	0.24	0.25	0.07	0.23

Table 18: Results of the individual non-compartmental pharmacokinetic analysis (75 mg/kg I.V.)
Unbound Concentrations in Lung

Time (min) \ Rat	**10B**	**11B**	**12B**	**13B**	**14B**	**15B**	**16B**	**Mean**	**S.D.**	**Median**
10	39.1	23.1	16.2	24.0	23.5	30.9	20.7	25.4	7.5	23.5
30	12.2	12.2	4.5	8.3	9.7	16.2	6.8	10.0	3.9	9.7
50	7.3	7.6	3.1	4.6	6.0	10.4	3.1	6.0	2.7	6.0
70	5.5	7.3	2.5	3.1	4.4	7.0	2.3	4.6	2.1	4.4
90	3.9	4.8	2.3	2.2	4.0	3.8	1.7	3.3	1.2	3.8
110	3.5	3.7	1.9	1.7	3.5	2.7	1.5	2.6	0.9	2.7
ke [min^{-1}]	0.013	0.013	0.007	0.016	0.009	0.023	0.013	0.013	0.005	0.013
n	4	4	4	4	4	4	4	4		4
$t_{1/2}$ [min]	54.2	52.9	95.0	42.4	80.6	30.0	54.6	58.5	22.2	54.2
C_{max} [μg/ml]	39.1	23.1	16.2	24.0	23.5	30.9	20.7	25.4	7.5	23.5
t_{max} [min]	10	10	10	10	10	10	10	10.0	0.0	10.0
AUC[μg/ml·min]	1469	1302	777	849	1277	1356	719	1107	312	1277
AUMC[μg/ml·min^2]	88252	92313	83067	40536	123509	61032	39193	75415	30434	83067
MRT[min]	60.1	70.9	106.9	47.8	96.7	45.0	54.5	68.8	24.2	60.1
F	0.29	0.25	0.23	0.30	0.19	0.38	0.31	0.28	0.06	0.29

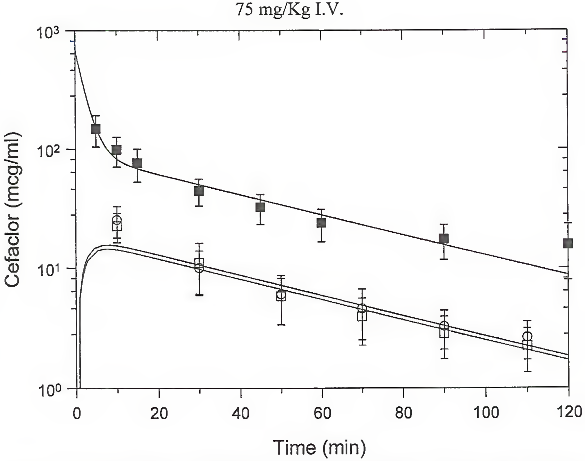


Figure 23: Mean plasma concentrations (± S.D.) of cefaclor (■) and mean unbound concentrations (± S.D.) in muscle (□) and lung (○). The lines represent the respective curve fits from nonlinear regression using simultaneous compartmental pharmacokinetics. (n=7)

Additional Experiments

Recovery by No Net Flux Method

Due to the variability in the results obtained by retrodialysis, the recovery was reassessed in muscle by the no net flux method in a separate set of 4 rats. Four different concentrations of cefaclor were perfused: 2, 4, 10 and 15 μg/mL, while keeping a constant tissue concentration of approximately 2.5 μg/mL. The recovery determined by this method was 33±20% and it was found not to be statistically significantly different from those found in both muscle and lung by retrodialysis (ANOVA, P>0.74). Figure 24 shows the plot for the no net flux method experiments.

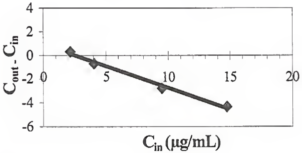


Figure 24: No net flux plot for cefaclor (n=4)

Continuous Infusion Experiment

From the animal experiments, it was observed that there is a difference between the free plasma concentrations and the free tissue concentrations of cefaclor. A continuous infusion experiment was performed to investigate if what was seen in the single-dose experiments would happen also in a steady-state situation.

A separate set of rats (n=3) were administered a loading dose of cefaclor (15 mg/kg) followed by a continuous IV infusion (0.3 mg/Kg/min). The same basic surgical procedure was used. Plasma samples were taken every 30 minutes and free muscle concentrations were obtained by microdialysis every 20 minutes for a total of 5 hours. Results are shown in Table 19 and Figure 25.

Table 19: Comparison between the single-dose and steady-state regimens.

Regimen	AUC free tissue conc./AUC plasma	AUC dialysates /AUC plasma
Single dose	0.20	0.08
Steady state	0.15	0.05

Areas under the curve were calculated for mean plasma, dialysates and free tissue concentrations. The ratios between the free tissue concentration AUC and dialysate AUC to the plasma AUC were compared for the different dosing regimens.

Figure 25 shows the cefaclor concentration-time profile for total and free plasma concentrations, and for free tissue concentrations, plotted as means ±S.D. of 3 rats.

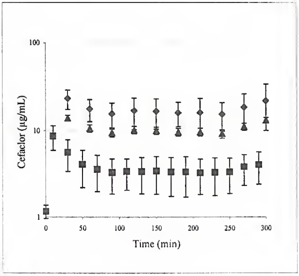


Figure 25: Mean (±S.D.) total plasma (◆), free plasma (▲) and free tissue (■) concentrations of cefaclor during continuous I.V. administration. (n=3)

Discussion

Cefaclor plasma concentrations were compared to unbound concentrations in muscle and lung as measured by microdialysis. Although both doses did not confirm dose-proportionality, for both doses, the measured concentrations in lung and muscle were virtually identical (AUC 1341 vs. 1108 µg/ml·min and 1011 vs. 1277 µg/ml·min, for muscle and lung, respectively). The half-life in plasma, muscle and lung was identical (56, 53 and 56 min. for 50 mg/kg and 59, 45 and 54 minutes for 75 mg/kg, in plasma, muscle and lung). Maximum concentrations reached in muscle and lung were 28.2 vs. 27.4 µg/ml and 20.6 vs. 23.5 µg/ml, hence also virtually identical. This study allows for the first time to directly compare the unbound concentrations of cefaclor in the muscle and the lung simultaneously. The results show that both tissues have equal concentrations representing 23-29% of unbound plasma concentrations, despite of highly variable plasma pharmacokinetics.

It was possible to describe the measured data with a pharmacokinetic two-compartment body model. Using a tissue distribution factor (or penetration factor), the measured muscle and lung concentrations could be well characterized by the unbound peripheral compartment concentrations of this model.

The most important conclusion from this study is the experimental confirmation, that unbound concentrations in muscle and lung are identical. If this result is applied to the clinical setting, it suggests that unbound concentrations measured in human muscle maybe reasonable predictors for therapeutically relevant unbound concentrations in human lung.

The difference between the free plasma concentration and the free tissue concentrations observed in the single-dose experiments was also observed in steady-state conditions, in the same order of magnitude. This behavior would be explained by either elimination from the peripheral compartment or by a saturable transport mechanism. The latter seems physiologically unlikely, more so when taking into account that in the single-dose experiments, the free concentrations in both tissues were identical. That would imply that if such a mechanism exists, it would have to be identical for both tissues. The inherent, well-documented instability of the drug is most probably the reason for this difference between free levels [211, 229, 230, 245, 246]. Cefaclor is a chemically unstable molecule that may be undergoing chemical degradation in the tissues faster than in blood. Further studies would be needed to address this issue.

CHAPTER 5
HUMAN PHARMACOKINETIC STUDIES

Aim of the Human Pharmacokinetic Studies

The objective of the study was to measure the concentration profile of cefaclor in skeletal muscle and in plasma following single oral administration of two different dosage forms of cefaclor in three different treatments (2 modified release, 1 immediate release) in healthy young subjects.

Materials and Methods

The study was a single center, open, randomized, three-way crossover, single dose study with a washout period of one week between the applications in healthy male volunteers.

The study population included 12 healthy male caucasian volunteers (age 25-35 years, weight 60-94 kg, height 170-191 cm). All 12 subjects completed the study. No replacements were done. The study was conducted at the University of Vienna, Department of Clinical Pharmacology, Allgemeines Krankenhaus, Währinger Gürtel 18-20, Vienna, Austria.

Two different dosage forms in three different treatments were compared:

1. 500 mg cefaclor in an immediate release dosage form (500 mg IR)
2. 500 mg cefaclor in a modified release dosage form (500 mg MR)
3. 750 mg cefaclor in a modified release dosage form (750 mg MR)

The data was analyzed both by non-compartmental and compartmental pharmacokinetic approaches.

The study was performed in accordance with the Declaration of Helsinki (revised version of Hong Kong, September 1989, latest German version published in BAnz. 108, June 1987, p. 1709), the Note for Guidance "Good Clinical Practice for Trials on Medicinal Products in the European Community" III/3957/88-EN of July 11, 1990 and the Austrian Arzneimittelgesetz (1994).

Experimental Design

Volunteers were given an entry examination not more than 14 days before the beginning of the trial. Each volunteer was subjected to a screening examination including history and physical examination, 12-lead ECG, complete blood count with differential, urinalysis, urine drug screen, clinical blood chemistry, blood coagulation test, HBs antigen and HIV antibody tests. All laboratory tests were carried out in the local laboratory of the clinical center. Subjects were excluded if they were taking any prescription medication or OTC drugs within a period of two weeks prior to the study. After determining the subject's suitability and prior to enrollment, written informed consent was obtained in response to a fully written and verbal explanation of the nature of the study.

Drug Administration

All volunteers received in each of the three periods of the trial one single oral dose of cefaclor 500 mg and 750 mg modified release and one single oral dose of cefaclor 500 mg immediate release. The time of the washout period between two applications was at least one week.

The study drug was administered according to a randomization scheme, after insertion of the microdialysis probe. The tablets were swallowed intact and were taken with 200 ml of tap water. The administration of the study medication was followed by a mouth check. A 7 days washout period followed.

Blood Sampling

The volunteers were in a supine position throughout the study period. A plastic cannula (Venflon®) was inserted into an antecubital vein for blood sampling before drug administration and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10 and 12 hours after drug administration.

Total blood loss over the whole study period was limited to a maximum of 300 ml per subject (7 ml per sample). Blood samples were centrifuged for 10 minutes at 3000 rpm (1000xg) and 4°C immediately after they were obtained and plasma was harvested in lithium-heparinised tubes. Plasma samples were frozen immediately and stored frozen at -70°C until analyzed.

Tissue Sampling

Unbound concentrations in thigh muscle were measured by microdialysis. Probe recovery was determined by retrodialysis, which allowed conversion of the measured dialysate concentrations to the actual unbound muscle concentrations.

The skin at the site of probe insertions was cleaned and disinfected. One dialysis probe was inserted into a medial vastus muscle by the following procedure: The surface of the skin was punctured by a 20-gauge I.V. plastic cannula. The steel mandrin was removed, and the dialysis probe was inserted into the plastic cannula. The microdialysis system was connected and perfused with Ringer´s solution at a flow rate of 1.5 µl/min. This was performed by a microinfusion-pump (Precidor®; Infors-AG, Basel, Switzerland). After a 30 minute baseline sampling period in vivo probe calibration was performed for a period of 30 minutes. Sampling times of dialysates were in 20 minutes intervals. Dialysate samples were frozen immediately and stored frozen at -70°C until analyzed. The probe was removed after the last blood sample was taken.

Estimation of Pharmacokinetic Parameters

Individual Non-compartmental Pharmacokinetic Analysis

The following parameters were calculated for each subject:

Plasma. The terminal elimination rate constant (k_e) was calculated by linear regression of the natural logarithms of the last n plasma concentrations. Terminal half-life was calculated as $\ln(2)/k_e$. The area under the curve (AUC) was calculated using the trapezoidal rule up to the last data point (C_X) and adding the extrapolated terminal area, calculated as C_X/k_e. The area under the first moment curve (AUMC) was calculated from a plot of $C \cdot t$ vs. t using the trapezoidal rule up to the last data point (C_X) at time t_x and adding the extrapolated terminal area, calculated as $C_X \cdot t_X/k_e + C_X/k_e^2$. The mean residence time (MRT) was calculated as AUMC/AUC. The highest concentration (C_{max}) was obtained directly from the experimental data, together with the respective time of maximum concentration (t_{max}).

Muscle. Unbound concentrations in muscle and lung were calculated from the measured microdialysate concentrations and the measured recovery from retrodialysis samples. The terminal elimination rate constant (k_e) was calculated by linear regression of the natural logarithms of the last n plasma concentrations. Terminal half-life was calculated as $\ln(2)/k_e$. The area under the curve (AUC) was calculated using the trapezoidal rule up to the last data point (C_X) and adding the extrapolated terminal area, calculated as C_X/k_e.

The area under the first moment curve (AUMC) was calculated from a plot of C·t vs. t using the trapezoidal rule up to the last data point (C_X) at time tx and adding the extrapolated terminal area, calculated as $C_X \cdot tx/k_e + C_X/k_e^2$. The mean residence time (MRT) was calculated as AUMC/AUC. The highest concentration (C_{max}) was obtained directly from the experimental data, together with the respective time of maximum concentration (tmax). The tissue penetration ratio (F) was calculated as the ratio of the unbound AUC in plasma and the unbound AUC in tissue (f_u·AUC/AUCT), where f_u is the fraction unbound in plasma.

Compartmental Pharmacokinetic Analysis

Immediate Release Dosage Form. For the compartmental pharmacokinetic data analysis of the plasma concentrations, a one-compartment body model with first order absorption and a lag-time was used. The respective equation for the plasma concentration C is

$$C = \frac{f \cdot D \cdot k_a}{(k_a - k_e) \cdot Vd} \cdot \left(e^{-k_e \cdot (t - t_{lag})} - e^{-k_a \cdot (t - t_{lag})}\right)$$

Equation 24

where D is the dose, f is the fraction absorbed, Vd is the volume of distribution, k_a is the absorption rate constant, k_e is the elimination rate constant, t_{lag} is the absorption lag time and t is time.

Unbound muscle concentrations (C_T) were fitted simultaneously with the respective plasma concentrations. The respective equation for the unbound concentration in the tissue is:

$$C_T = \frac{F \cdot f_u \cdot f \cdot D \cdot k_{am}}{(k_{am} - k_e) \cdot Vd} \cdot \left(e^{-k_e \cdot (t - t_{lag})} - e^{-k_{am} \cdot (t - t_{lag})}\right)$$

Equation 25

where F is the penetration ratio factor, f_u the fraction unbound in plasma and k_{am} the absorption rate constant based on muscle concentrations.

Modified Release Dosage Forms. For the compartmental pharmacokinetic data analysis of the plasma concentrations, a two-compartment body model with time-limited zero order absorption and a lag-time was used. The respective equation for the plasma concentration C is

$$C = \frac{k_0}{V_c} \cdot \left[\frac{\left(1 - e^{\alpha \cdot T}\right) \cdot \left(k_{21} - \alpha\right)}{\alpha \cdot (\alpha - \beta)} \cdot e^{-\alpha \cdot \left(t - t_{lag}\right)} + \frac{\left(1 - e^{\beta \cdot T}\right) \cdot \left(k_{21} - \beta\right)}{\beta \cdot (\beta - \alpha)} \cdot e^{-\beta \cdot \left(t - t_{lag}\right)} \right]$$

Equation 26

where k_0 is the zero-order absorption rate, V_c is the volume of distribution of the central compartment, α is the hybrid rate constant for distribution, β is the hybrid constant for elimination, k_{21} is the first-order rate constant for the transfer from the peripheral into the central compartment, t_{lag} is the absorption lag time, T is the absorption time that stops at time t=tstop and t is time.

Unbound muscle concentrations (C_T) were fitted simultaneously with the respective plasma concentrations. The respective differential equation for the amount of drug in the peripheral compartment (X_T) is:

$$\frac{dX_T}{dt} = k_{12} \cdot C \cdot V_c - k_{21} \cdot X_T$$

Equation 27

where k_{12} is the first-order rate constant for the transfer from the central into the peripheral compartment. From this equation, the unbound concentration in the peripheral compartment (C_T) can be calculated as:

$$C_T = \frac{X_T \cdot f_u \cdot F}{\left(Vd_{ss} - V_c\right)}$$

Equation 28

where F is the penetration ratio, f_u the fraction unbound in plasma and Vd_{ss} the volume of distribution at steady state.

The data was analyzed by nonlinear regression using the program Scientist (MicroMath, Salt Lake City, Utah). For each subject, the plasma concentrations were fitted to both models. The coefficient of determination (CD) as well as the Model Selection Criterion (MSC) were used as a criterion for the goodness of the resulting curve fits. The closer CD is to 1, the better the agreement between measured and calculated values. The higher MSC, the more appropriate the selected model.

Recovery of Microdialysis probes

To obtain interstitial concentrations from dialysate concentrations, microdialysis probe calibration was assessed according to the retrodialysis method. The principle of this method relies on the assumption that the diffusion process is quantitatively equal in both directions through the semipermeable membrane. Therefore, the study drug was added to the perfusion medium in a concentration of 10µg/ml and the disappearance rate through the membrane was taken as the in vivo recovery. The in vivo recovery value was calculated as:

$$R(\%) = \frac{C_{in} - C_{out}}{C_{in}} \cdot 100$$

Equation 29

where R is the recovery in percent, C_{in} is the known concentration being perfused, and C_{out} is the concentration of the dialysate.

Results

Individual Non-compartmental Pharmacokinetic Analysis

Plasma. The results of the individual non-compartmental pharmacokinetic data analysis for cefaclor in plasma are listed in Table 20 (500 mg IR), Table 21 (500 mg MR) and Table 22 (750 mg MR).

Figure 26 shows a comparison of the mean concentrations measured in plasma for the three treatments and their respective standard deviations.

For the 500 mg IR treatment, mean maximum concentrations were 12.7 µg/ml observed after 1.2 hours. The average terminal half-life was 0.8 hours. The mean area under the curve was 17.6 µg/ml. The mean residence time was 1.7 hours.

For the 500 mg MR treatment, mean maximum concentrations were 4.7 µg/ml observed after 2.1 hours. The average terminal half-life was 0.8 hours. The mean area under the curve was 13.7 µg/ml. The mean residence time was 2.9 hours.

For the 750 mg MR treatment, mean maximum concentrations were 7.2 µg/ml observed after 2.2 hours. The average terminal half-life was 0.7 hours. The mean area under the curve was 22.1 µg/ml. The mean residence time was 2.7 hours.

Table 20: Results of the individual non-compartmental pharmacokinetic analysis (500 mg IR); Plasma

	Volunteer																	
T(h)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Mean**	**S.D.**	**C.V.**	**Min**	**Max**	**Median**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0%	0.00	0.00	0.00
0.5	13.54	1.09	2.06	1.35	17.05	0.45	1.03	16.99	1.08	1.57	5.88	0.80	5.24	6.61	126%	0.45	17.05	1.46
1	12.28	6.68	9.07	11.50	10.51	14.36	11.38	7.04	15.31	8.82	10.95	1.20	9.93	3.78	38%	1.20	15.31	10.73
1.5	8.52	7.91	9.41	6.35	4.86	11.74	10.30	3.63	6.83	12.22	6.93	2.71	7.62	3.01	40%	2.71	12.22	7.42
2	3.55	8.40	5.49	3.29	2.19	6.15	6.89	1.98	3.21	6.55	3.51	3.13	4.53	2.08	46%	1.98	8.40	3.53
2.5	1.95	4.62	2.61	2.02	1.17	3.57	3.55	1.04	1.62	3.62	1.56	11.11	3.20	2.73	85%	1.04	11.11	2.31
3	0.95	2.13	1.33	2.25	0.71	2.27	2.34	0.66	0.86	1.88	0.93	7.56	1.99	1.88	94%	0.66	7.56	1.61
3.5	0.52	1.04	0.77	2.20	0.45	1.43	0.99	0.48	0.58	0.48	0.38	3.11	1.04	0.84	81%	0.38	3.11	0.67
4	0.24	0.32	0.25	1.08	0.29	0.94	0.65	0.41	0.23	0.69	0.41	1.42	0.58	0.39	67%	0.23	1.42	0.41
5	<LOD	0.10	<LOD	<LOD	0.15	0.42	0.13	<LOD	<LOD	0.26	0.17	0.20	0.20	0.11	52%	0.10	0.42	0.17
6	<LOD	<LOD	<LOD	<LOD	<LOD	0.31	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	NC	NC	NC	0.31	0.31	0.31
k_e [h^{-1}]	1.391	1.574	1.508	0.379	0.823	0.718	1.322	0.623	1.256	1.011	0.861	1.647	1.093	0.414	38%	0.38	1.65	1.13
n	4	5	4	4	5	6	5	4	4	5	5	5	4.7	0.7	14%	4.00	6.00	5.00
$t_{1/2}$ [h]	0.50	0.44	0.46	1.83	0.84	0.96	0.52	1.11	0.55	0.69	0.81	0.42	0.76	0.40	53%	0.42	1.83	0.62
AUC [µg/ml·h]	20.9	16.3	15.6	17.6	18.9	21.7	18.9	16.7	15.0	18.5	15.7	16.1	17.6	2.2	12%	14.98	21.70	17.13
AUMC [µg/ml·h^2]	25.3	30.3	25.2	43.8	21.0	41.7	33.1	19.7	21.7	33.0	22.6	42.1	30.0	8.8	29%	19.66	43.84	27.79
MRT[h]	1.2	1.9	1.6	2.5	1.1	1.9	1.8	1.2	1.4	1.8	1.4	2.6	1.7	0.5	28%	1.11	2.61	1.68
C_{max} [µg/ml]	13.5	8.4	9.4	11.5	17.0	14.4	11.4	17.0	15.3	12.2	11.0	11.1	12.7	2.8	22%	8.40	17.05	11.86
t_{max}[h]	0.5	2.0	1.5	1.0	0.5	1.0	1.0	0.5	1.0	1.5	1.0	2.5	1.2	0.6	53%	0.50	2.50	1.00

<LOD= lower than limit of detection; NS = no sample available for assay; NC=not calculated; NR=not reportable due to system malfunction

Table 21: Results of the individual non-compartmental pharmacokinetic analysis (500 mg MR); Plasma

	Volunteer																	
T (h)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Mean**	**S.D.**	**C.V.**	**Min**	**Max**	**Median**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0%	0.00	0.00	0.00
0.5	3.15	0.00	0.13	0.00	1.48	0.00	0.51	2.54	0.61	0.00	2.70	0.67	0.98	1.18	120%	0.00	3.15	0.56
1	4.16	0.48	3.17	0.96	2.33	4.61	3.58	1.33	0.89	1.41	3.23	1.03	2.26	1.43	63%	0.48	4.61	1.87
1.5	3.60	0.23	3.59	0.56	2.43	3.75	3.91	2.42	1.13	4.28	4.08	2.29	2.69	1.41	53%	0.23	4.28	3.01
2	3.44	3.54	3.65	1.44	1.85	3.77	3.20	3.57	4.72	4.53	4.36	2.86	3.41	0.99	29%	1.44	4.72	3.55
2.5	3.58	6.85	3.49	6.36	1.55	4.08	2.18	3.73	5.19	3.65	4.24	2.19	3.92	1.61	41%	1.55	6.85	3.69
3	3.00	4.64	3.06	5.53	0.98	3.09	1.94	3.77	2.18	3.09	3.41	5.70	3.37	1.40	41%	0.98	5.70	3.09
3.5	3.46	3.46	1.55	5.02	1.23	3.15	1.65	3.22	0.83	2.82	3.69	6.80	3.07	1.68	55%	0.83	6.80	3.19
4	3.11	2.70	0.71	4.28	1.16	3.37	1.19	2.66	3.54	2.34	2.33	3.84	2.60	1.12	43%	0.71	4.28	2.68
5	1.69	1.34	0.19	1.53	0.35	2.48	1.52	1.58	1.09	0.74	0.82	1.96	1.28	0.66	52%	0.19	2.48	1.43
6	0.41	0.40	<LOD	0.49	<LOD	0.79	0.62	0.42	0.37	0.19	0.21	0.34	0.42	0.18	42%	0.19	0.79	0.41
k_e [h^{-1}]	0.847	0.859	1.370	0.965	0.882	0.551	0.894	0.802	1.130	1.111	1.140	1.149	0.975	0.216	22%	0.55	1.37	0.93
n	4	4	3	4	3	4	2	4	3	4	4	4	3.6	0.7	19%	2.00	4.00	4.00
$t_{1/2}$ [h]	0.82	0.81	0.51	0.72	0.79	1.26	0.78	0.86	0.61	0.62	0.61	0.60	0.75	0.20	26%	0.51	1.26	0.75
AUC [µg/ml·h]	16.9	13.6	10.1	15.4	7.4	18.1	11.9	14.6	12.0	12.7	15.7	16.1	13.7	3.1	22%	7.37	18.07	14.12
AUMC [µg/ml·h^2]	46.6	44.9	23.0	52.8	17.6	60.2	34.5	43.6	36.8	35.1	39.6	52.8	40.6	12.3	30%	17.59	60.25	41.57
MRT[h]	2.8	3.3	2.3	3.4	2.4	3.3	2.9	3.0	3.1	2.8	2.5	3.3	2.9	0.4	13%	2.28	3.43	2.94
C_{max} [µg/ml]	4.2	6.9	3.6	6.4	2.4	4.6	3.9	3.8	5.2	4.5	4.4	6.8	4.7	1.4	29%	2.43	6.85	4.44
t_{max}[h]	1	2.5	2	2.5	1.5	1	1.5	3	2.5	2	2	3.5	2.1	0.8	37%	1.00	3.50	2.00

<LOD= lower than limit of detection; NS = no sample available for assay; NC=not calculated; NR=not reportable due to system malfunction

Table 22: Results of the individual non-compartmental pharmacokinetic analysis (750 mg MR); Plasma

	Volunteer																	
T (h)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Mean**	**S.D.**	**C.V.**	**Min**	**Max**	**Median**
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0%	0.00	0.00	0.00
0.5	4.64	5.76	0.95	0.68	3.59	1.74	2.34	0.74	0.00	1.64	0.21	8.13	2.53	2.52	99%	0.00	8.13	1.69
1	3.67	5.09	5.97	2.89	4.40	1.78	5.25	6.48	3.15	NS	0.81	6.58	4.19	1.91	46%	0.81	6.58	4.40
1.5	5.14	6.79	3.45	4.08	5.42	5.49	4.34	6.26	7.54	4.87	3.21	6.46	5.25	1.35	26%	3.21	7.54	5.28
2	5.18	5.02	5.88	5.08	6.24	6.70	4.03	4.68	8.45	4.70	8.01	5.74	5.81	1.35	23%	4.03	8.45	5.46
2.5	4.99	4.13	6.60	8.54	5.35	8.10	2.87	6.76	6.38	5.84	6.47	6.34	6.03	1.57	26%	2.87	8.54	6.36
3	5.91	4.39	6.72	8.11	5.78	7.83	2.22	5.91	5.48	6.16	6.38	5.38	5.86	1.53	26%	2.22	8.11	5.91
3.5	6.83	4.14	5.05	6.70	5.13	5.84	3.00	4.54	3.58	4.10	6.50	3.73	4.93	1.30	26%	3.00	6.83	4.79
4	5.63	2.11	3.58	4.04	2.88	3.43	2.67	4.30	2.96	2.84	4.34	2.08	3.40	1.04	30%	2.08	5.63	3.19
5	2.15	0.08	0.37	1.18	0.71	1.19	4.54	2.93	0.65	0.86	1.84	0.43	1.41	1.28	91%	0.08	4.54	1.02
6	0.34	0.08	0.19	0.28	0.14	1.18	1.74	1.04	0.32	0.21	0.53	<LOD	0.55	0.53	98%	0.08	1.74	0.32
k_e [h^{-1}]	1.206	1.741	1.440	1.276	1.448	0.669	0.960	0.586	1.048	1.209	0.998	1.453	1.169	0.337	29%	0.59	1.74	1.21
n	4	4	4	4	4	4	2	4	4	4	4	3	3.8	0.6	17%	2.00	4.00	4.00
$t_{1/2}$ [h]	0.57	0.40	0.48	0.54	0.48	1.04	0.72	1.18	0.66	0.57	0.69	0.48	0.65	0.24	36%	0.40	1.18	0.57
AUC [μg/ml·h]	25.0	19.4	20.6	22.6	21.0	24.9	21.3	26.1	20.6	18.6	21.7	23.3	22.1	2.3	11%	18.55	26.13	21.47
AUMC [μg/ml·h^2]	69.7	40.4	52.9	64.7	51.0	77.2	71.4	82.4	53.3	49.1	67.4	47.5	60.6	13.2	22%	40.38	82.37	58.98
MRT[h]	2.8	2.1	2.6	2.9	2.4	3.1	3.4	3.2	2.6	2.6	3.1	2.0	2.7	0.4	15%	2.04	3.36	2.72
C_{max} [μg/ml]	6.8	6.8	6.7	8.5	6.2	8.1	5.2	6.8	8.4	6.2	8.0	8.1	7.2	1.1	15%	5.25	8.54	6.81
t_{max}[h]	3.5	1.5	3	2.5	2	2.5	1	2.5	2	3	2	0.5	2.2	0.9	40%	0.50	3.50	2.25

<LOD= lower than limit of detection; NS = no sample available for assay; NC=not calculated; NR=not reportable due to system malfunction

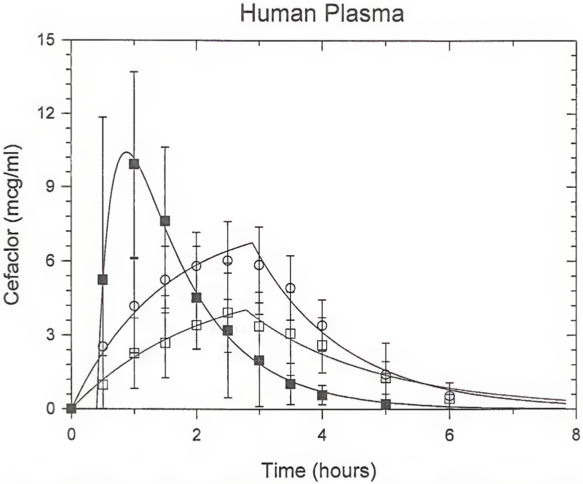
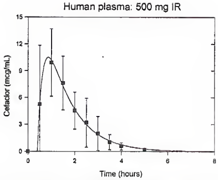


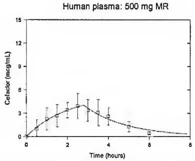
Figure 26: Mean plasma concentrations (± S.D.) of cefaclor in plasma after administration of 500 mg IR (■), 500 mg MR (□) and 750 mg MR (○). The lines represent the curve fits from the respective compartmental data analysis.

The individual plots are shown in next page (figures 1-A, B and C.)

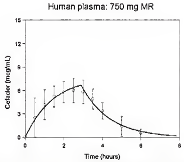
Figure 26 cont.


Human plasma: 500 mg IR

A: Mean plasma concentrations (± S.D.) of cefaclor in plasma after administration of 500 mg IR (■). The lines represent the curve fits from the respective compartmental data analysis.


Human plasma: 500 mg MR

B: Mean plasma concentrations (± S.D.) of cefaclor in plasma after administration of 500 mg MR (□). The lines represent the curve fits from the respective compartmental data analysis.


Human plasma: 750 mg MR

C: Mean plasma concentrations (± S.D.) of cefaclor in plasma after administration of 750 mg MR (○). The lines represent the curve fits from the respective compartmental data analysis.

Muscle. The results of the individual non-compartmental pharmacokinetic data analysis for cefaclor for the unbound concentrations in muscle are listed in Table 23 to Table 25. The results are based on an average recovery of 22.8%. The median values for the cefaclor concentrations in the retrodialysis solution and samples used for different subjects were calculated. The recovery value was then calculated as the average percent deviation of the retrodialysis samples from the retrodialysis solution.

Figure 27 shows a comparison of the mean unbound concentrations in muscle measured for the three treatments and their respective standard deviations.

For the 500 mg IR treatment, mean maximum unbound muscle concentrations were 6.9 µg/ml observed after 1.4 hours. The average terminal half-life was 0.7 hours. The mean area under the curve was 9.49 µg/ml·h. The mean residence time was 1.9 hours. The tissue penetration ratio F was 0.73.

For the 500 mg MR treatment, mean maximum concentrations were 2.5 µg/ml observed after 2.3 hours. The average terminal half-life was 0.8 hours. The mean area under the curve was 7.02 µg/ml·h. The mean residence time was 3.5 hours. The tissue penetration ratio F was 0.67.

For the 750 mg MR treatment, mean maximum concentrations were 4.2 µg/ml observed after 2.6 hours. The average terminal half-life was 0.7 hours. The mean area under the curve was 11.53 µg/ml·h. The mean residence time was 3.0 hours. The tissue penetration ratio F was 0.70.

Table 23: Results of the individual non-compartmental pharmacokinetic analysis (500 mg IR); Unbound Extracellular Muscle Fluid

	Volunteer																	
T (h)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Mean**	**S.D.**	**C.V.**	**Min**	**Max**	**Median**
0.00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.00	0.00	0%	0.00	0.00	0.00
0.17	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.00	0.00	0%	0.00	0.00	0.00
0.50	2.676	0.000	0.439	0.562	1.953	1.320	0.231	5.681	0.000	0.455	0.804	0.254	1.20	1.63	136%	0.00	5.68	0.51
0.83	6.626	0.000	4.283	3.099	7.517	1.261	2.265	8.848	2.631	2.794	3.456	0.622	3.62	2.75	76%	0.00	8.85	2.95
1.17	4.192	2.521	8.701	4.515	4.921	6.198	6.696	4.196	5.786	5.752	6.419	1.042	5.08	2.01	40%	1.04	8.70	5.34
1.50	2.948	4.918	8.391	3.628	3.246	5.581	5.947	3.239	4.553	8.683	6.105	1.451	4.89	2.18	45%	1.45	8.68	4.74
1.83	2.425	4.897	5.893	2.220	1.824	3.290	5.390	1.521	2.436	5.268	4.977	1.906	3.50	1.65	47%	1.52	5.89	2.86
2.17	1.023	6.384	4.058	1.495	1.271	2.647	3.634	1.193	1.695	2.688	2.712	1.671	2.54	1.56	61%	1.02	6.38	2.17
2.50	0.764	2.900	1.896	0.657	0.981	1.501	2.935	0.719	0.985	1.932	2.290	4.492	1.84	1.17	63%	0.66	4.49	1.70
2.83	0.296	2.796	0.776	0.981	0.781	1.074	2.033	0.682	0.767	1.461	1.136	6.182	1.58	1.60	101%	0.30	6.18	1.03
3.17	<LOD	1.123	0.513	1.037	0.607	0.726	1.802	0.566	0.464	0.708	0.590	6.774	1.36	1.84	136%	0.46	6.77	0.71
3.50	<LOD	0.984	0.593	1.038	<LOD	0.585	1.037	<LOD	0.449	0.559	0.566	3.383	1.02	0.92	90%	0.45	3.38	0.59
3.83	<LOD	0.464	<LOD	0.862	<LOD	0.441	0.695	<LOD	<LOD	<LOD	0.205	2.403	NC	NC	NC	0.21	2.40	0.58
4.17	<LOD	0.589	<LOD	0.508	<LOD	0.229	0.506	<LOD	<LOD	0.284	<LOD	0.915	NC	NC	NC	0.23	0.91	0.51
4.50	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.596	<LOD	<LOD	<LOD	0.175	1.199	NC	NC	NC	0.18	1.20	0.60
4.83	<LOD	<LOD	<LOD	<LOD	<LOD	0.176	0.372	<LOD	<LOD	<LOD	<LOD	NR	NC	NC	NC	0.18	0.37	0.27
5.17	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.309	NC	NC	NC	0.31	0.31	0.31
k_e [h^{-1}]	1.981	1.199	1.171	0.450	0.733	1.122	0.661	0.761	1.023	1.244	1.657	1.431	1.119	0.437	39%	0.45	1.98	1.15
n	4	5	4	5	4	4	5	5	4	5	5	5	4.6	0.5	11%	4.00	5.00	5.00
$t_{1/2}$ [h]	0.35	0.58	0.59	1.54	0.95	0.62	1.05	0.91	0.68	0.56	0.42	0.48	0.73	0.33	46%	0.35	1.54	0.60
AUC [µg/ml·h]	7.08	9.58	12.26	7.91	8.43	8.54	11.88	9.53	6.95	10.52	9.95	11.28	9.49	1.77	19%	6.95	12.26	9.56
AUMC [µg/ml·h^2]	8.89	21.89	20.78	19.42	13.94	15.63	26.00	13.52	12.23	18.84	17.10	33.24	17.14	25.51	149%	8.89	33.24	17.97
MRT[h]	1.3	2.3	1.7	2.5	1.7	1.8	2.2	1.4	1.8	1.8	1.7	2.9	1.9	0.5	25%	1.25	2.95	1.78
C_{max} [µg/ml]	6.6	6.4	8.7	4.5	7.5	6.2	6.7	8.8	5.8	8.7	6.4	6.8	6.9	1.3	19%	4.52	8.85	6.66
t_{max}[h]	0.8	2.2	1.2	1.2	0.8	1.2	1.2	0.8	1.2	1.5	1.2	3.2	1.4	0.7	50%	0.83	3.17	1.17
F	0.45	0.78	1.05	0.60	0.59	0.52	0.84	0.76	0.62	0.76	0.85	0.93	0.73	0.18	24%	0.45	1.05	0.76

<LOD= lower than limit of detection; NS = no sample available for assay; NC=not calculated; NR=not reportable due to system malfunction

Table 24: Results of the individual non-compartmental pharmacokinetic analysis (500 mg MR); Unbound Extracellular Muscle Fluid

	Volunteer																	
T (h)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Mean**	**S.D.**	**C.V.**	**Min**	**Max**	**Median**
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.32	0.00	0.00	0.00	0.00	0.03	0.09	346%	0.00	0.32	0.00
0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.40	0.00	0.00	0.00	0.34	0.06	0.15	234%	0.00	0.40	0.00
0.50	0.65	0.00	0.00	0.00	0.00	0.00	0.00	0.86	0.00	0.00	0.00	0.00	0.14	0.31	225%	0.00	0.86	0.00
0.83	1.23	0.00	0.37	0.00	NS	0.00	0.59	0.68	0.41	0.00	1.55	0.65	0.50	0.52	105%	0.00	1.55	0.41
1.17	1.20	0.00	1.75	0.38	0.65	1.16	2.35	1.17	0.48	NS	1.35	0.65	1.12	0.61	55%	0.38	2.35	1.17
1.50	1.11	0.00	2.17	0.00	0.57	1.95	2.48	0.68	0.49	1.05	1.97	0.96	1.12	0.84	75%	0.00	2.48	1.00
1.83	0.65	0.78	1.84	0.38	0.19	1.71	3.28	1.96	0.64	2.04	2.20	1.18	1.40	0.92	65%	0.19	3.28	1.44
2.17	0.62	1.06	1.85	0.51	<LOD	2.09	1.87	1.03	1.63	1.82	2.44	1.61	1.50	0.62	41%	0.51	2.44	1.63
2.50	0.68	2.58	1.64	1.94	0.44	0.98	1.94	2.91	2.01	1.81	1.98	1.44	1.70	0.72	43%	0.44	2.91	1.87
2.83	1.00	2.61	1.88	3.00	<LOD	2.39	1.15	1.56	1.93	1.48	2.28	1.21	1.86	0.65	35%	1.00	3.00	1.88
3.17	0.79	2.47	1.27	2.81	<LOD	1.39	2.06	3.03	1.14	1.48	2.08	1.43	1.81	0.73	40%	0.79	3.03	1.48
3.50	1.04	1.25	0.93	2.63	<LOD	1.36	1.12	1.76	0.86	1.49	1.94	3.19	1.60	0.74	46%	0.86	3.19	1.36
3.83	0.73	1.70	0.54	2.51	<LOD	1.46	1.21	2.34	1.53	1.29	1.35	2.44	1.56	0.66	42%	0.54	2.51	1.46
4.17	0.97	1.26	0.49	2.10	<LOD	1.80	0.67	0.92	1.70	0.77	1.33	2.10	1.28	0.57	45%	0.49	2.10	1.26
4.50	1.12	1.85	<LOD	1.47	<LOD	2.05	1.07	1.72	1.23	0.83	0.65	1.61	1.36	0.46	34%	0.65	2.05	1.35
4.83	0.86	1.27	<LOD	1.09	<LOD	2.27	0.76	1.10	0.91	0.36	0.89	1.78	1.13	0.54	48%	0.36	2.27	1.00
5.17	0.50	0.80	<LOD	0.80	<LOD	0.82	1.30	1.07	0.44	0.48	<LOD	1.04	0.80	0.30	37%	0.44	1.30	0.80
5.50	0.20	<LOD	<LOD	0.57	<LOD	0.83	0.74	0.75	0.32	<LOD	0.28	0.82	0.56	0.26	46%	0.20	0.83	0.65
5.83	<LOD	<LOD	<LOD	0.50	<LOD	0.63	1.00	0.56	<LOD	<LOD	<LOD	0.32	NC	NC	NC	0.32	1.00	0.56
6.17	<LOD	<LOD	<LOD	<LOD	<LOD	0.57	<LOD	<LOD	<LOD	<LOD	<LOD	0.31	NC	NC	NC	0.31	0.57	0.44
6.50	<LOD	<LOD	<LOD	<LOD	<LOD	0.34	0.58	<LOD	<LOD	<LOD	<LOD	<LOD	NC	NC	NC	0.34	0.58	0.46
6.83	<LOD	<LOD	<LOD	<LOD	<LOD	0.32	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	NC	NC	NC	0.32	0.32	0.32
7.17	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.24	<LOD	<LOD	<LOD	<LOD	<LOD	NC	NC	NC	0.24	0.24	0.24
k_e [h^{-1}]	1.701	0.451	1.070	0.845	NC	0.765	1.122	0.786	1.307	0.823	0.927	1.402	1.018	0.350	34%	0.45	1.70	0.93
n	4	5	5	5	NC	5	3	5	5	5	5	5	4.7	0.6	14%	3.00	5.00	5.00
$t_{1/2}$ [h]	0.41	1.54	0.65	0.82	NC	0.91	0.62	0.88	0.53	0.84	0.75	0.49	0.77	0.30	40%	0.41	1.54	0.75
AUC [µg/ml·h]	4.54	7.51	5.28	7.40	NC	8.41	8.72	8.78	5.43	5.47	7.88	7.84	7.02	1.54	22%	4.54	8.78	7.51
AUMC [µg/ml·h^2]	12.99	32.38	13.23	28.35	NC	31.05	41.66	29.69	18.03	17.98	22.49	27.45	12.37	13.33	108%	12.99	41.66	27.45
MRT[h]	2.9	4.3	2.5	3.8	NC	3.7	4.8	3.4	3.3	3.3	2.9	3.5	3.5	0.7	19%	2.51	4.78	3.38
C_{max} µg/ml]	1.2	2.6	2.2	3.0	NC	2.4	3.3	3.0	2.0	2.0	2.4	3.2	2.5	0.6	25%	1.23	3.28	2.44
t_{max}[h]	0.8	2.8	1.5	2.8	NC	2.2	1.8	3.2	2.5	2.5	2.2	3.5	2.3	0.8	33%	0.83	3.50	2.50
F	0.36	0.73	0.70	0.64	NC	0.62	0.98	0.80	0.60	0.58	0.67	0.65	0.67	0.15	23%	0.36	0.98	0.65

<LOD= lower than limit of detection; NS = no sample available for assay; NC=not calculated; NR=not reportable due to system malfunction

Table 25: Results of the individual non-compartmental pharmacokinetic analysis (750 mg MR); Unbound extracellular muscle fluid

T (h)	1	2	3	4	5	6	7	8	9	10	11	12	Mean	S.D.	C.V.	Min	Max	Median
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0%	0.00	0.00	0.00
0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.19	NS	0.00	0.00	0.02	0.06	332%	0.00	0.19	0.00
0.50	0.74	1.46	0.00	0.00	0.70	0.38	0.43	0.00	0.18	0.00	0.00	NR	0.35	0.47	131%	0.00	1.46	0.18
0.83	1.45	3.32	1.23	0.35	1.30	1.83	1.64	0.63	0.00	4.00	0.00	4.17	1.66	1.45	88%	0.00	4.17	1.38
1.17	1.76	3.46	1.67	0.00	1.77	2.53	3.84	NS	1.02	4.56	0.00	NR	2.06	1.55	75%	0.00	4.56	1.77
1.50	1.53	3.84	1.58	1.03	2.65	2.64	2.14	NS	2.62	2.43	1.19	NS	2.17	0.85	39%	1.03	3.84	2.29
1.83	NR	3.21	1.27	1.39	3.74	3.10	2.14	NS	6.82	2.80	2.20	NR	2.96	1.67	56%	1.27	6.82	2.80
2.17	2.09	2.82	2.12	1.91	4.26	3.69	2.41	3.68	4.00	2.43	3.40	2.83	2.97	0.81	27%	1.91	4.26	2.83
2.50	NR	3.36	2.15	2.48	2.73	3.94	1.95	5.64	4.57	2.65	2.96	4.36	3.34	1.15	34%	1.95	5.64	2.96
2.83	1.94	2.08	2.55	2.87	2.31	4.52	1.40	5.19	4.23	3.53	2.85	3.17	3.05	1.13	37%	1.40	5.19	2.86
3.17	NR	3.47	2.37	2.93	2.40	4.30	1.34	5.17	4.28	3.40	1.94	2.31	3.08	1.16	38%	1.34	5.17	2.93
3.50	2.49	0.57	2.36	2.56	4.96	3.60	1.44	3.62	1.92	2.46	3.43	1.36	2.56	1.20	47%	0.57	4.96	2.47
3.83	NR	1.96	1.23	2.95	2.75	2.89	1.64	4.25	2.97	1.20	1.27	1.41	2.23	1.00	45%	1.20	4.25	1.96
4.17	2.08	1.88	1.17	1.65	1.98	1.52	1.28	2.59	2.01	1.40	2.20	1.27	1.75	0.44	25%	1.17	2.59	1.77
4.50	1.75	0.71	0.80	0.91	1.14	1.03	1.90	3.62	1.85	0.92	1.30	<LOD	1.45	0.84	58%	0.71	3.62	1.14
4.83	1.31	<LOD	0.76	0.67	0.96	0.87	1.73	2.83	0.69	0.64	1.50	0.91	1.17	0.66	56%	0.64	2.83	0.91
5.17	1.11	0.52	0.49	0.35	0.42	0.59	2.44	3.09	0.70	0.28	0.83	<LOD	0.98	0.92	94%	0.28	3.09	0.59
5.50	0.68	<LOD	0.43	<LOD	0.31	0.14	2.64	1.54	0.44	0.39	0.63	0.39	0.76	0.76	101%	0.14	2.64	0.43
5.83	0.59	<LOD	<LOD	0.28	0.22	0.46	2.31	1.26	0.31	<LOD	<LOD	<LOD	NC	NC	NC	0.22	2.31	0.46
6.17	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.22	0.56	<LOD	<LOD	0.32	<LOD	NC	NC	NC	0.32	1.22	0.56
6.50	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.08	0.70	<LOD	<LOD	<LOD	<LOD	NC	NC	NC	0.70	1.08	0.89
6.83	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.69	0.30	<LOD	<LOD	<LOD	<LOD	NC	NC	NC	0.30	0.69	0.49
7.17	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.35	0.44	<LOD	<LOD	<LOD	<LOD	NC	NC	NC	0.35	0.44	0.40
7.83	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.20	<LOD	<LOD	<LOD	<LOD	<LOD	NC	NC	NC	0.20	0.20	0.20
k_e [h^{-1}]	0.852	1.096	0.752	1.084	1.334	1.029	1.177	0.822	1.215	1.115	0.926	1.252	1.054	0.183	17%	0.75	1.33	1.09
n	5	4	5	5	5	5	5	5	5	5	5	5	4.9	0.3	6%	4.00	5.00	5.00
$t_{1/2}$ [h]	0.81	0.63	0.92	0.64	0.52	0.67	0.59	0.84	0.57	0.62	0.75	0.55	0.68	0.13	19%	0.52	0.92	0.64
AUC[µg/ml·h]	9.88	11.48	7.90	7.75	11.66	13.05	12.30	17.65	13.11	11.32	10.56	11.72	11.53	2.59	23%	7.75	17.65	11.57
AUMC [µg/ml·h^2]	32.49	28.02	24.31	25.36	32.71	37.26	44.88	63.68	37.80	28.55	31.01	28.72	21.54	56.04	260%	24.31	63.68	31.75
MRT[h]	3.3	2.4	3.1	3.3	2.8	2.9	3.7	3.6	2.9	2.5	2.9	2.4	3.0	0.4	14%	2.44	3.65	2.91
C_{max}[µg/ml]	2.5	3.8	2.6	3.0	5.0	4.5	3.8	5.6	6.8	4.6	3.4	4.4	4.2	1.3	31%	2.49	6.82	4.10
t_{max}[h]	3.5	1.5	2.8	3.8	3.5	2.8	1.2	2.8	1.8	1.2	3.5	2.5	2.6	1.0	37%	1.17	3.83	2.83
F	0.53	0.79	0.51	0.46	0.74	0.70	0.77	0.90	0.85	0.81	0.65	0.67	0.70	0.14	20%	0.46	0.90	0.72

<LOD= lower than limit of detection; NS = no sample available for assay; NC=not calculated; NR=not reportable due to system malfunction

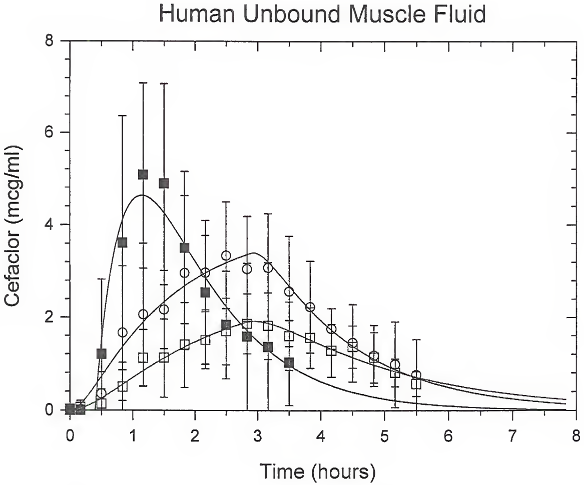
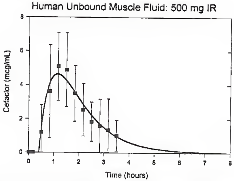


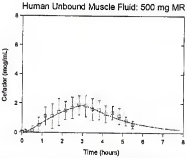
Figure 27: Mean plasma concentrations (± S.D.) of unbound cefaclor in muscle after administration of 500 mg IR (■), 500 mg MR (□) and 750 mg MR (○). The lines represent the curve fits from the respective compartmental data analysis.

The individual plots are shown in next page (figures 2-A, B and C)

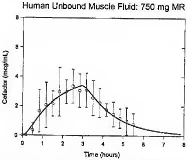
Figure 27 cont.



A: Mean plasma concentrations (± S.D.) of unbound cefaclor in muscle after administration of 500 mg IR (■). The lines represent the curve fits from the respective compartmental data analysis.



B: Mean plasma concentrations (± S.D.) of unbound cefaclor in muscle after administration of 500 mg MR (□). The lines represent the curve fits from the respective compartmental data analysis.



C: Mean plasma concentrations (± S.D.) of unbound cefaclor in muscle after administration of 750 mg MR (○). The lines represent the curve fits from the respective compartmental data analysis.

Compartmental Pharmacokinetic Analysis

Immediate Release Product (500 mg IR).

A one-compartment model with first-order absorption and a lag time was able to produce a good curve fit of the average plasma concentrations after oral administration of the 500 mg IR product (Figure 28). The model also allowed a good curve fit of the respective unbound muscle concentrations. The coefficient of determination (r^2) was 0.989, the model selection criterion (MSC) was 4.0, indicating good curve fits.

The respective parameters are shown in Table 26. As can be seen from Figure 28, the model describes both plasma and unbound muscle concentrations very well.

Table 26: Compartmental pharmacokinetic parameters, 500 mg IR

Parameter	500 mg IR (Mean ± S.D.)
r^2	0.989
MSC	4.0
k_a (h^{-1})	3.9 ± 0.93
k_e (h^{-1})	0.94 ± 0.09
Vd/f	30.5 ± 2.2
t_{lag}	0.40 ± 0.02
k_{am}	1.85 ± 0.31
F	0.76 ± 0.03

Modified Release Product (500 mg MR).

A two-compartment model with zero-order absorption was able to produce a good curve fit of the average plasma concentrations after oral administration of the 500 mg MR product (Figure 29). The model also allowed a good curve fit of the respective unbound muscle concentrations. The coefficient of determination (r^2) was 0.975, the model selection criterion (MSC) was 3.2, indicating reasonable curve fits.

The respective parameters are shown in Table 27. As can be seen from Figure 29, the model describes both plasma and unbound muscle concentrations very well.

Modified Release Product (750 mg MR).

A two-compartment model with zero-order absorption was able to produce a good curve fit of the average plasma concentrations after oral administration of the 750 mg MR product (Figure 30). The model also allowed a good curve fit of the respective unbound muscle concentrations. The coefficient of determination (r^2) was 0.984, the model selection criterion (MSC) was 3.7, indicating good curve fits.

The respective parameters are shown in Table 27.

As can be seen from Figure 30, the model describes both plasma and unbound muscle concentrations very well.

Table 27: Compartmental pharmacokinetic parameters, 500 mg and 750 mg MR

Parameter	500 mg MR (Mean ± S.D.)	750 mg MR (Mean ± S.D.)
r^2	0.975	0.984
MSC	3.2	3.7
k_{12} (h^{-1})	0.10 ± 1.07	0.10 ± 1.38
k_{21} (h^{-1})	2.29 ± 0.55	4.23 ± 1.19
k_{10} (h^{-1})	0.50 ± 0.22	0.71 ± 0.23
Vc/f (L)	65.2 ± 29.4	47.0 ± 15.6
t_{stop} (h)	2.8 ± 0.1	2.9 ± 0.1
F	0.68 ± 0.03	0.69 ± 0.02

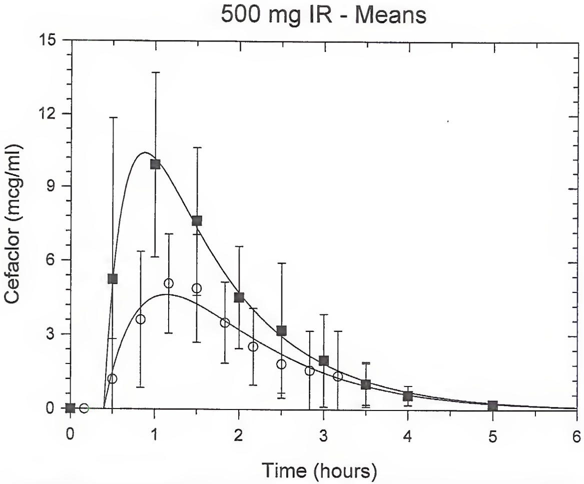


Figure 28: Mean concentrations (± S.D.) of cefaclor in plasma (■) as well as unbound cefaclor concentration in muscle (○) after administration of 500 mg IR. The lines represent the curve fits from the respective compartmental data analysis.

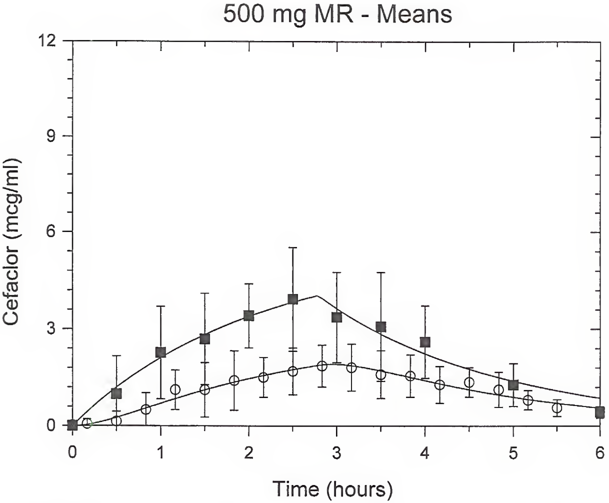


Figure 29: Mean concentrations (± S.D.) of cefaclor in plasma (■) as well as unbound cefaclor concentration in muscle (○) after administration of 500 mg MR. The lines represent the curve fits from the respective compartmental data analysis.

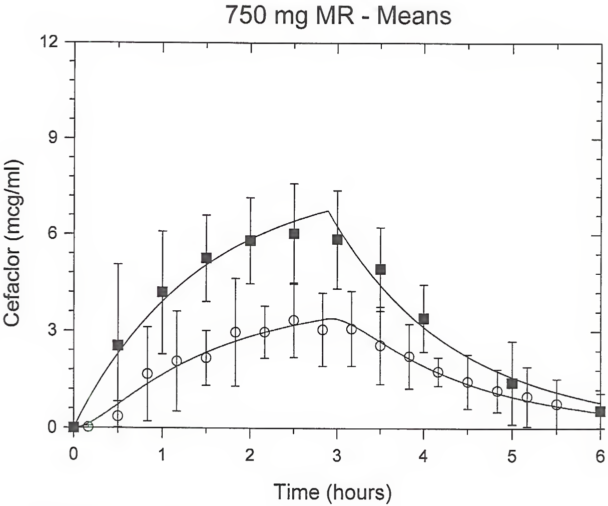


Figure 30: Mean concentrations (± S.D.) of cefaclor in plasma (■) as well as unbound cefaclor concentration in muscle (○) after administration of 750 mg MR. The lines represent the curve fits from the respective compartmental data analysis.

Recovery of Microdialysis Probes

Table 28: In vivo recoveries for volunteers 1-6 for all treatment days

Recovery (%)	Volunteer					
Day	01/RS	02/HS	03/HS	04/CS	05/CM	06/OS
1	16.2	8.7	60.0	12.6	23.1	-2.3
2	16.8	56.1	56.7	19.5	25.9	31.6
3	26.1	16.5	2.93	20.4	23.3	-0.3

Table 29: In vivo recoveries for volunteers 7-12 for all treatment days

Recovery (%)	Volunteer					
Day	07/AJ	08/TK	09/AS	10/KK	11/RH	12/MS
1	25.7	20.8	14.3	42.9	34.7	19.6
2	46.9	41.3	-2.2	28.1	24.5	-4.9
3	22.2	41.9	18.4	45.2	29.4	31.3

Table 30: Human in vivo recovery calculated using the medians of the measurements

	Median	SD	CV%
Retro sol	943120	150650	16%
R1	777885	186293	24%
R2	678015	122509	18%
Mean	727950		
Recovery	22.8%		

R1 and R2 are the two microdialysis samples collected while perfusing the 10µg/mL cefaclor retro solution at the beginning of the experiment. The negative values are not plausible, that is why the medians of all the values were used to calculate the recovery instead of the means.

Discussion

In plasma, mean maximum concentrations were 12.7, 4.7 and 7.2 μg/ml observed after 1.2, 2.1 and 2.2 hours, for 500 mg IR, 500 mg MR and 750 mg MR, respectively. The average terminal half-life was 0.7-0.8 h for all three treatments. The mean area under the curve was 17.6, 13.7 and 22.1 μg/ml; the mean residence times increased from 1.7 h to 2.9 and 2.7 h in plasma for the MR formulation. The relative bioavailability of the 500 mg and 750 MR products in comparison to the 500 mg IR was 78% and 84%, respectively.

Statistical analysis was performed using SigmaStat® for Windows v.2.0 (Jandel Corporation, 1995). The predetermined significance level (alpha) was set to 0.05 in all cases. In plasma, a 1-way repeated measures ANOVA found significant differences between the treatments for AUC and C_{max} ($p<0.001$ in both cases). A multiple comparisons procedure (Tukey's test) showed all three treatments to be different from each other for both AUC and C_{max} ($p<0.05$ in both cases). A 1-way repeated measures ANOVA found significant differences between the treatments for MRT and t_{max} ($p<0.001$ and $p=0.012$ respectively) and Tukey's test found them to be significantly longer for the MR formulations ($p<0.05$ in both cases). No statistically significant difference between treatments was found for plasma $t_{1/2}$.

Measuring unbound muscle concentrations by microdialysis allows to assess the concentration-time profiles in the peripheral tissue compartment. In vivo average microdialysis probe recovery in muscle was 22.8%. This recovery was employed to convert measured microdialysate concentrations into unbound muscle concentrations.

The measured profiles mirror those in plasma. In muscle, maximum concentrations were lower than in plasma: 6.9, 2.5 and 4.2 μg/ml observed after 1.9, 2.3 and 2.6 hours, for 500 mg IR, 500 mg MR and 750 mg MR, respectively. The average terminal half-life was 0.7-0.8 h for all three treatments, hence consistent with plasma. The mean area under the curve was 9.5, 7.0 and 11.5 μg/ml·h; the mean residence times in muscle increased from 1.9 h for the IR product to 3.5 and 3.0 h for the two MR products. Unbound concentrations in tissue reached approximately 70% of unbound concentrations in plasma based on the respective areas under the curves.

In unbound tissue levels, a 1-way repeated measures ANOVA found significant differences between the treatments for AUC, C_{max} and MRT ($p \leq 0.001$ in all cases). A multiple comparisons procedure (Tukey's test) showed all three treatments to be different from each other for all parameters ($p \leq 0.05$ in all cases). The AUC for both 500 mg formulations should theoretically be identical if there are no differences in absorption. The absorption for the modified release formulation is limited due to its slower liberation from the formulation, the limited surface area available for β-lactam antibiotic absorption ("absorption window"), and the gastrointestinal transit time. Thus, for both MR formulations, the absorption is seen to stop at approximately 3 hours, which corresponds to the time that the drug is going through the small intestine and during which it can be absorbed. The implications of this fact are that the MR dose is not completely absorbed, and thus the AUC for the 500 mg MR formulation is lower than the AUC for the 500 mg IR formulation, which is in turn comparable to the 750 mg MR formulation.

A 1-way repeated measures ANOVA found significant differences between the treatments for t_{max} ($p=0.003$) and Tukey's test found it to be significantly longer for the

MR formulations ($p<0.05$). No statistically significant difference between treatments was found for unbound tissue $t_{1/2}$ or for the tissue penetration factor F (power = 74% and 53% respectively).

It was possible to identify suitable pharmacokinetic models to describe both IR and MR products consistently. Whereas the IR product shows rapid first-order absorption after a 20-30 min. absorption lag, the MR products show constant zero-order absorption over a time period of approximately 3 hours, when absorption ceases. In the developed pharmacokinetic models, it was possible to link the measured unbound muscle concentrations to the measured plasma levels. This will allow to use the pharmacokinetic parameters in combination with suitable pharmacodynamic data to set up PK-PD models for rational dosing exploration.

CHAPTER 6
PHARMACODYNAMIC STUDIES

Aim of the Pharmacodynamic Studies

The aim of the pharmacodynamic studies was to investigate the *in vitro* effect of cefaclor on four different bacterial strains: Escherichia coli ATCC® 25922, Moraxella catarrhalis ATCC® 25240, Haemophilus influenzae ATCC® 10211 and Streptococcus pneumoniae ATCC® 49619. Based on the results of the human pharmacokinetic studies, the concentration-time profile of free cefaclor concentrations in tissue after administration of commonly used doses was simulated in an *in vitro* model. Bacteria were exposed to these simulated cefaclor concentrations and the change in the number of bacteria vs. time was monitored. Fitting of the data using a mathematical model resulted in a set of parameters for each bacterial strain, to be subsequently used in the simulation of effects for different dosing regimens.

Materials and Methods

Drug

Cefaclor (CCL) reference standard was provided by ACS DOBFAR (Hamburg, Germany) via Hexal AG (Holzkirchen, Germany). The compound was stored in a refrigerator in the original opaque vial. Cefaclor stock solutions were prepared in distilled water and aliquots frozen at –70°C for later use.

Bacteria

Four different bacterial strains were used as test strains: Escherichia coli ATCC® 25922 (E. coli), Moraxella catarrhalis ATCC® 25240 (M. cat), Haemophilus influenzae ATCC® 10211 (H. flu) and Streptococcus pneumoniae ATCC® 49619 (S. pneumo). All bacteria were obtained from the Microbiology Lab, Shands Hospital, University of Florida.

Determination of Minimum Inhibitory Concentrations

The cefaclor minimum inhibitory concentrations (MICs) for the four bacterial strains were determined using the broth macrodilution method and taken into account for the calculation of the Cefaclor doses [10]. The minimum inhibitory concentration is defined as the lowest concentration of drug inhibiting visible macroscopic growth [270]. Table 31 shows the MIC values obtained for the four bacterial strains. An additional penicillin resistant Streptococcus pneumoniae clinical isolate (Microbiology Lab., Shands Hospital, University of Florida) was added for comparison.

Table 31: Cefaclor MIC values and respective bacterial growth media

Bacterial Strain	**Growth media**	**MIC (µg/mL)**
Escherichia coli ATCC® 25922	Mueller-Hinton	3.13 - 6.25
Moraxella catarrhalis ATCC® 25240	Mueller-Hinton	< 0.2
Haemophilus influenzae ATCC® 10211	Haemophilus Test Media	<0.4 - 1.6
Penicillin Sensitive Streptococcus pneumoniae ATCC® 49619	Todd Hewitt	1.6 - 6.25
Penicillin Resistant Streptococcus pneumoniae (clinical isolate)	Todd Hewitt	100

The results obtained in this study agree with the reported MIC range values for E. coli, M. catarrhalis and H. influenzae [255, 271]. The MIC value for the penicillin sensitive strain of S. pneumoniae are higher than those reported in the literature [255, 271]. As expected, the MIC value for the penicillin resistant strain of S. pneumoniae is higher than that of the penicillin sensitive strain.

Broth Preparation

Mueller-Hinton Broth and Todd Hewitt broth were both prepared according to the manufacturer's instructions and autoclaved prior to use at 121°C (40 minutes per 500 mL). Haemophilus Test Media (HTM) Broth was purchased sterile and ready to use and stored in a refrigerator at approximately 7°C.

The broth types used were Mueller-Hinton for E. coli and M. catarrhalis, Haemophilus Test Media (HTM) for H. influenzae, Todd Hewitt for S. pneumoniae.

In vitro Model of Infection

A one-compartment *in vitro* model of infection was used to simulate the free tissue concentrations of cefaclor based on human tissue pharmacokinetic data. Bacteria were exposed to different concentrations of cefaclor.

The *in vitro* infection model consists of a 50-mL canted neck, vented cap tissue culture flask containing 20 mL of the appropriate growth media. A syringe connected to a sterile 0.2 µm filter was adapted to the upper side of the flask to allow the withdrawal and replacement of broth solution for the simulation of decreasing concentrations. In order to reproduce the one-hour half-life of cefaclor in humans, a stepwise dilution procedure was followed. At 15 minute intervals, 2.5 mL of the media were withdrawn and replaced by fresh sterile broth solution. The *in vitro* model was kept at 37°C during the experiment.

In another set of experiments, the bacteria were exposed to a high concentration of cefaclor without the dilution steps, and the maximum kill curve was determined as described above.

Microorganism Inoculation

The inoculum was prepared from colonies incubated overnight on appropriate agar plates. The microorganisms were suspended in sterile normal saline to a concentration equivalent to a 0.5 value in the McFarland Equivalence Turbidity Standard (Remel Microbiology Products, Lenexa, KS) ($1x10^8$ CFU/mL) as measured with a turbidimeter. Using a micropipette, 5µL of the suspension were inoculated into the *in vitro* model to achieve a final inoculum of approximately $5x10^5$ CFU. The model was incubated for 2 hours to allow the bacteria to reach the logarithmic growth phase prior to the addition of Cefaclor.

Cefaclor Doses

After the incubation period, a time zero sample was taken and the Cefaclor dose was added to the model. In the case of the 2-dose regimen simulation, the second dose was given at 4 hours and the experiment continued up to 8 hours. Table 32 shows the initial concentrations of cefaclor the bacteria were exposed to in the *in vitro* model.

Table 32: Initial cefaclor concentrations

	Dose I		**Dose II**	**Maximum kill**
Bacteria	**1-dose regimen**	**2-dose regimen**	**1-dose regimen**	**1-dose regimen**
E. coli	5 µg/mL	5 µg/mL	20 µg/mL	50 µg/mL
M. cat	0.5 µg/mL	0.5 µg/mL	5 µg/mL	50 µg/mL
H. flu	20 µg/mL	20 µg/mL	5 µg/mL	50 µg/mL
S. pneumo	5 µg/mL	5 µg/mL	20 µg/mL	50 µg/mL

Bacterial Quantification

Samples (50 μL) were collected from the in vitro model at times 0 and every hour up to 6 hours for the one dose regimen; and at times 0 and every hour up to 8 hours for the two dose regimen. Bacterial counts were determined by plating three serial 10- or 100-fold dilutions of the samples on appropriate agar plates. The dilutions were made in sterile normal saline. Aliquots (100 μL) of each dilution were plated in duplicate. Five % sheep blood agar plates were used for E. coli, M. catarrhalis and S. pneumoniae [272]. Chocolate agar plates were used for H. influenzae [272]. The plates were incubated at 37°C and 5% CO_2 for 24 hours before reading.

The procedure was repeated at least 4 times per bacterial strain and dose. Positive controls with bacteria but no drug were run simultaneously. Negative controls (no bacteria, no drug) were used for at least one run per bacterial strain to assess the method.

Following incubation, colonies were counted in all readable plates that showed up to approximately 300 colonies. The number of colonies in each dilution tube at each time was determined by averaging the counts obtained. The data was entered in Microsoft Excel spreadsheets. The numbers of colonies were adjusted to an initial concentration of $1x10^5$CFU/mL. The mean values (± 1 standard deviation) were plotted against time.

Results

Kill Curves

The following tables (Table 33 to Table 36) show the results of the experiments in CFU/mL for each bacterial strain, expressed as mean±S.D. Number of replicates are indicated in each case. Figure 31 to Figure 34 show the corresponding plots of CFU/mL vs. time for the different concentrations the bacteria were exposed to.

Table 33: CFU/mL vs. time; Escherichia coli ATCC® 25922

Time (h)	5 µg/mL (n=4)	5µg/mL, 2-d (n=4)	20µg/mL (n=4)	50µg/mL (n=3)	+ controls (n=8)
0	1.0E+05	1.0E+05	1.0E+05	1.0E+05	1.0E+05
1	(2.3±1.8) E+05	(3.5±1.7)E+04	(1.6±1.5)E+04	(2.3±1.5)E+03	(5.6±4.1)E+05
2	(6.9±2.9)E+04	(2.4±2.0)E+04	(1.9±1.5)E+03	1.0E+03	(8.7±10)E+06
3	(4.2±3.5)E+04	(4.7±2.0)E+04	(1.4±0.99)E+03	NC	(6.2±3.0)E+07
4	(8.3±6.6)E+04	(5.1±6.5)E+05	(4.4±4.2)E+02	NC	(2.4±1.1)E+08
5	(4.7±3.3)E+05	(3.7±3.9)E+05	(4.8±5.0)E+03	ND	(7.9±7.5)E+08
6	(8.3±11)E+06	(6.8±3.5)E+05	(3.2±3.1)E+04	ND	(8.0±2.0)E+08
7	ND	(9.7±3.7)E+05	ND	ND	1.0E+09*
8	ND	(3.7±1.2)E+06	ND	ND	2.3E+09*

NC= non-countable in any dilution, ND = not done; *only 1 replicate (no S.D.)

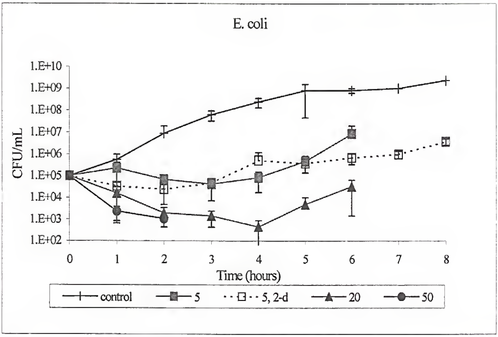


Figure 31: Escherichia coli: Number of bacteria as a function of time for various initial cefaclor concentrations. Concentrations are expressed in µg/mL.

Table 34: CFU/mL vs. time; Moraxella catarrhalis ATCC® 25240

Time (h)	0.5 μg/mL (n=4)	0.5 μg/mL, 2-d (n=4)	5 μg/mL (n=4)	50 μg/mL (n=3)	+ controls (n=11)
0	1.0E+05	1.0E+05	1.0E+05	1.0E+05	1.0E+05
1	(6.2±2.8)E+04	(8.5±6.7)E+04	(7.7±8.6)E+04	(1.3±0.7)E+04	(3.7±3.5)E+05
2	(5.6±4.0)E+04	(5.3±3.0)E+04	(5.0±3.3)E+04	(8.3±8.2)E+03	(6.4±3.3)E+05
3	(2.2±1.0)E+04	(3.9±1.0)E+04	(4.0±3.0)E+04	(8.1±2.9)E+03	(9.4±11)E+05
4	(2.0±0.8)E+04	(8.9±8.3)E+04	(2.3±2.2)E+04	(8.4±5.6)E+03	(2.7±2.7)E+06
5	(6.3±5.5)E+04	(4.4±1.6)E+04	(4.2±3.3)E+04	ND	(3.8±3.4)E+06
6	(1.0±0.6)E+05	(2.6±2.8)E+04	(3.0±1.2) E+04	ND	(1.6±1.5)E+07
7	ND	(2.1±0.9)E+04	ND	ND	(2.8±3.0)E+07
8	ND	(1.6±0.6)E+04	ND	ND	2.2E+06*

ND= not done; * only 1 replicate (no S.D., not plotted)

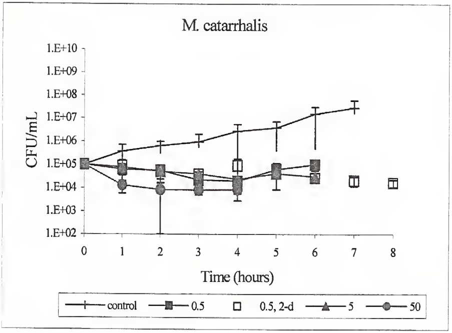


Figure 32: Moraxella catarrhalis: Number of bacteria as a function of time for various initial cefaclor concentrations. Concentrations are expressed in μg/mL.

Table 35: CFU/mL vs. time; Haemophilus influenzae ATCC® 10211

Time (h)	20 µg/mL (n=4)	20 µg/mL, 2-d (n=4)	5 µg/mL (n=4)	50 µg/mL (n=3)	+ controls (n=6)
0	1.0E+05	1.0E+05	1.0E+05	1.0E+05	1.0E+05
1	(7.4±4.7)E+04	(1.3±0.3) E+05	(1.2±1.0)E+05	(6.7±2.6)E+04	(2.2±1.4)E+05
2	(5.5±2.0)E+04	(1.1±0.5)E+05	(1.7± 0.1)E+05	(5.7±2.0)E+04	(6.3±4.9)E+05
3	(3.0±2.2)E+04	(1.1±0.2)E+05	(3.4±1.6)E+05	(4.2±3.5)E+04	(1.6±1.7)E+06
4	(5.0±3.5)E+04	(9.2±2.6)E+04	(1.1±1.1)E+06	(1.5±1.6)E+04	(4.3±2.8)E+06
5	(4.7±0.9)E+04	(8.6±2.0)E+04	(1.8±1.3)E+06	ND	(1.1±1.4)E+07
6	(1.4±1.1)E+05	(1.1±0.3)E+05	(6.5±2.4)E+06	ND	(4.2±4.5)E+07
7	ND	(1.0±0.2)E+05	ND	ND	1.4E+05*
8	ND	(1.2±0.6)E+05	ND	ND	2.1E+05*

ND= not done, * only 1 replicate (no S.D., not plotted)

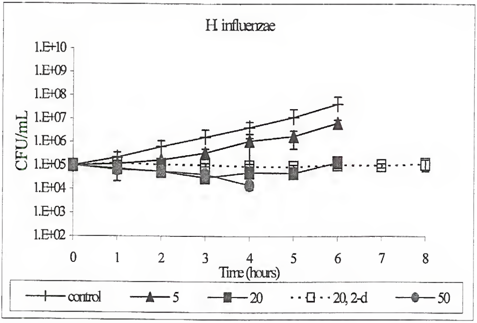


Figure 33: Haemophilus influenzae: Number of bacteria as a function of time for various initial cefaclor concentrations. Concentrations are expressed in µg/mL.

Table 36: CFU/mL vs. time; Streptococcus pneumoniae ATCC® 49619

Time (h)	5 µg/mL (n=4)	5 µg/mL, 2-d (n=4)	20 µg/mL (n=4)	50 µg/mL (n=3)	+ controls (n=6)
0	1.0E+05	1.0E+05	1.0E+05	1.0E+05	1.0E+05
1	(2.3±2.9)E+05	(1.7±0.5)E+05	(1.3±0.2)E+05	(9.1±3.6)E+04	(3.9±2.4)E+05
2	(1.2±0.8)E+05	(1.4±0.9)E+05	(6.3±5.9)E+04	(4.2±0.8)E+04	(1.9±1.4)E+06
3	(9.7±8.7)E+04	(9.8±4.1)E+04	(1.4E±0.5)E+04	(8.1±5.1)E+03	(1.4±0.6)E+07
4	(1.0±1.4)E+05	(6.9±1.2)E+04	(1.3±0.5)E+04	(3.2±2.3)E+03	(5.8±3.8)E+07
5	(5.4±2.3)E+04	(5.7±4.6)E+04	(3.5±3.3)E+03	ND	(2.6±1.4)E+08
6	(2.0±2.8)E+05	(3.4±3.5)E+04	(2.2±1.1)E+03	ND	(3.8±2.3)E+08
7	ND	(1.7±2.0)E+04	ND	ND	1.1E+09*
8	ND	(2.2±2.4)E+04	ND	ND	4.1E+08*

ND= not done, * only 1 replicate (no S.D.)

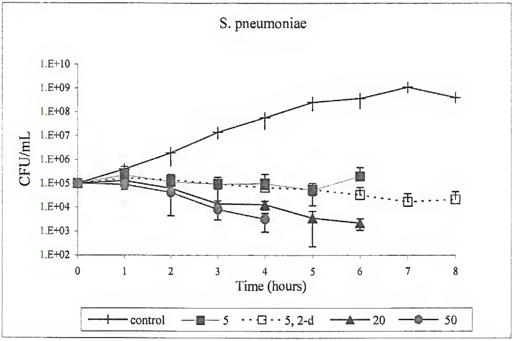


Figure 34: Streptococcus pneumoniae: Number of bacteria as a function of time for various initial cefaclor concentrations. Concentrations are expressed in µg/mL.

Times Above MIC

The times above MIC were calculated taking into account the measured MICs for the bacterial strains and the unbound tissue concentrations reached in the human study (see Chapter 5) and are shown below in Table 37 for a single dose regimen. In the cases where bacterial MICs were reported as a range, the lower limit of the range was used.

Table 37: Time above MIC (hours) for unbound tissue concentrations of cefaclor.

	Time above MIC (hours) (Mean ± S.D., n=12)			
	Bacteria			
Dose	E. coli	M. catarrhalis	H. influenzae	S. pneumoniae
500 mg IR	1.05±0.31	3.55±0.70	3.39±0.55	1.80±0.36
500 mg MR	0.66[a]	4.79±1.54	4.36±1.36	1.96±0.48
750 mg MR	1.37±0.68[b]	5.25±1.00	4.86±0.74	3.08±0.55

a Only in one subject were the concentrations above the E. coli MIC for a 500 mg MR dose (n=1);

b Only in 9 subjects were the concentrations above the E. coli MIC for a 750 mg MR dose (n=9)

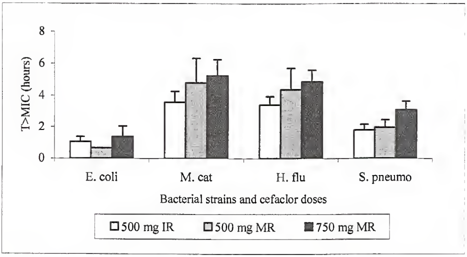


Figure 35: Time above MIC (hours) of the studied bacterial strains for human unbound tissue concentrations due to the different cefaclor dosing regimens. The bars represent the mean results, error bars show the S.D. (n=12). No S.D. is shown for E. coli at 500 mg MR because only in one subject the concentrations were above the MIC.

Times above MIC (t>MIC) for total plasma concentrations were also calculated as described above, results are shown below in Table 38. The t>MIC in plasma were compared to the t>MIC for the unbound tissue concentrations. Figure 36 shows the results for the 4 bacterial strains studied.

Table 38: Times above MIC (hours) for plasma concentrations of cefaclor.

	Time above MIC (hours) (Mean ± S.D., n=12)			
	Bacteria			
Dose	E. coli	M. catarrhalis	H. influenzae	S. pneumoniae
500 mg IR	1.75±0.26	4.33±0.65	3.88±0.43	2.38±0.43
500 mg MR	2.09±0.63	5.46±0.78	5.13±0.80	3.13±1.00
750 mg MR	3.27±0.41	5.59±0.66	5.08±0.70	4.17±0.72

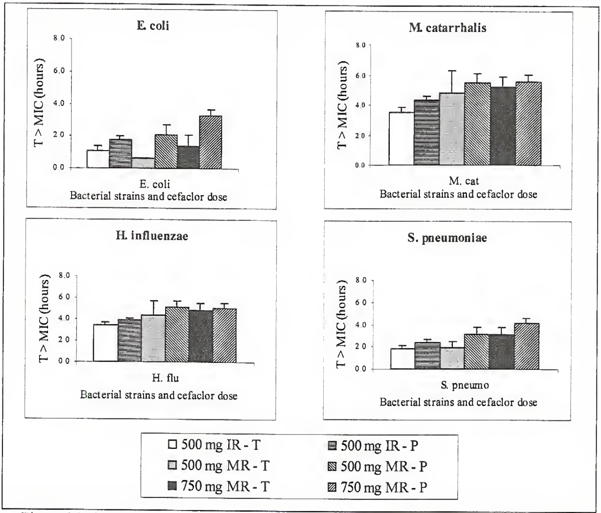


Figure 36: Comparison of the t>MIC for plasma and unbound tissue concentrations in each bacterial strain studied, for each cefaclor dose. The bars represent the t>MIC for the different cefaclor doses, for T = unbound tissue concentrations (solid) and P = plasma concentrations (striped).

From the values presented for unbound tissue concentrations in Table 37, the percent of time that the concentrations are above the MIC in a 24-hour period were calculated for qd (once every 24 hours), bid (once every 12 hours) and tid (once every 8 hours) administrations. Results are shown in Table 39 and Figure 37.

Table 39: Percent t > MIC in 24 hours for unbound tissue concentrations of cefaclor, calculated for qd, bid and tid regimens.

	E. coli			M. cat			H. flu			S. pneumo		
	qd	bid	tid	qd	bid	tid	qd	bid	tid	qd	bid	tid
500 mg IR	4%	9%	13%	15%	30%	44%	14%	28%	42%	8%	15%	23%
500 mg MR	3%	6%	8%	20%	40%	60%	18%	36%	54%	8%	16%	25%
750 mg MR	6%	11%	17%	22%	44%	66%	20%	40%	61%	13%	26%	39%

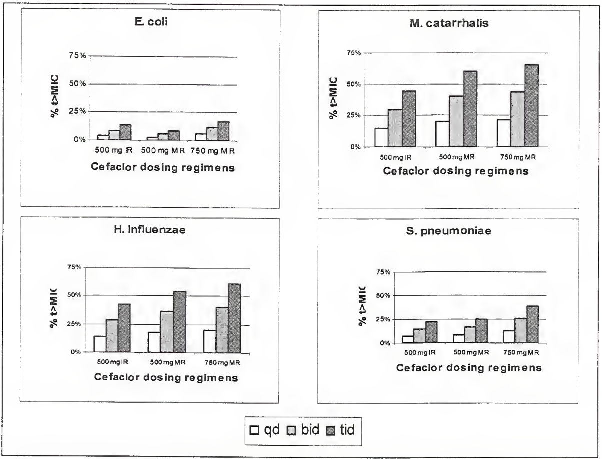


Figure 37: Comparison between the percent of the time per day that concentrations are above the MIC (%t>MIC) for unbound tissue concentrations of cefaclor. Each plot represents one bacterial strain. qd = every 24 hours, bid = every 12 hours, tid = every 8 hours.

Discussion

The *in vitro* model used enabled to follow the bacterial kill and regrowth curves. An increase in the effect with an increase in initial cefaclor concentration up to a maximum initial concentration of 50 μg/mL was observed for E. coli (Figure 31). In the case of M. catarrhalis, curves due to initial concentrations of 0.5 μg/mL and 5.0 μg/mL were almost superimposable, but at higher concentrations (50 μg/mL) the effect was seen to increase again (Figure 32). The curves for H. influenzae (Figure 33) show a very small effect at 5 μg/mL, which is seen to increase at an initial concentration of 20μg/mL. Not much difference is seen between the 20μg/mL and the 50μg/mL curves, which indicates that at 20μg/mL we are probably already approaching the maximum kill rate for this bacterial strain. A similar effect is observed for S. pneumoniae (Figure 34), with the two highest concentrations following very similar profiles.

The results of the t>MIC data calculated from the unbound tissue concentrations, for a qd administration (Table 37, Figure 35) were analyzed using SigmaStat® v.2.0 (Jandel Scientific, 1995). A one-way ANOVA showed significant differences between all three treatments for M. catarrhalis ($p=0.003$), S. pneumoniae ($p=0.001$), and H.influenzae ($p=0.002$). The data was further analyzed by means of a multiple comparison procedure (Tukey's test). For both M. catarrhalis and S. pneumoniae, the MR formulations showed significant differences compared to the IR formulation ($p<0.05$), but no differences between themselves ($P>87\%$). For S. pneumoniae there were significant differences between the 750 mg MR and both the 500 mg IR and 500 mg MR doses ($p<0.05$), while the 500 mg doses showed no differences between themselves ($P=100\%$). Due to the lack of data for E. coli for the 500 mg MR treatment, it was not taken into account, and the

500 mg IR was compared to the 750 mg MR using a t-test that failed to find differences between them.

Times above MIC were calculated also for the plasma concentrations. They were found to be higher than those from unbound tissue concentrations for every dose and bacteria studied. Thus, using t>MIC derived from plasma, as it is traditionally done, would consistently overestimate the effectiveness of the drug (Figure 36).

Finally, the t>MIC were calculated from the clinically relevant, unbound tissue concentrations for the case of once, twice and three times a day administration, for the different cefaclor doses and all the bacteria studied. Results are expressed as the percent of time that the unbound tissue concentrations are above the MIC in 24 hours (Table 39, Figure 37). According to these results, a tid administration of the 500 mg formulation would be identical to a bid administration of the 750 mg formulation, even though the latter delivers a smaller total amount of drug due to its incomplete absorption. The maximum %t>MIC for the unbound tissue concentrations (17%, 66%, 61% and 39% for E.coli, M. catarrhalis, H. influenzae and S. pneumoniae respectively) would be achieved with a tid administration of the 750 mg MR formulation. Craig et al. recommend that β-lactam concentrations should be above the MIC for at least 40% of the dosing interval to achieve bacteriological cure [21]. According to this theory a tid administration of 750 mg MR cefaclor should be effective against all the bacteria studied, with the exception of E. coli.

CHAPTER 7
PHARMACOKINETIC-PHARMACODYNAMIC MODELING

Data Analysis

The mean values of the experimental data for each dose regimen and each bacterial strain were simultaneously fitted to the following model using the non-linear least-square regression program Scientist® v.2.01 (Micromath®):

$$\frac{dN}{dt} = \left(k_0 - \frac{k_{\max} \cdot C}{EC_{50} + C} \right) \cdot N$$

Equation 30

where dN/dt is the change in the number of bacteria as a function of time, k_0 (h^{-1}) is the bacterial growth rate constant in the absence of antibiotic, k_{max} (h^{-1}) is the maximum killing rate constant (maximum effect), C (µg/mL) is the concentration of cefaclor at time t, and EC_{50} (µg/mL) is the concentration of cefaclor necessary to produce 50% of the maximum effect. The pharmacodynamic parameters for the different bacteria were thus obtained, and are shown in Table 40 to Table 43. Subsequently, the term C was substituted for the expressions for the human unbound tissue concentrations of the different cefaclor formulations and doses shown in Chapter 5. The resulting equations were utilized together with the appropriate parameters for each dose, formulation and bacteria, to generate the simulations of the effects of the named dosage regimens on the bacterial strains. The results of the simulations are shown in Figure 42 to Figure 45.

Results

The results of the pharmacodynamic data analysis are listed in the following tables:

Table 40: Pharmacodynamic parameters; E. coli

	Initial Cefaclor concentrations (µg/mL)					
Parameters	50	20	5; 1-d	5; 2-d	Mean	S.D.
k_{max} (h^{-1})	6.4	6.0	5.9	6.1	6.1	0.2
k_0 (h^{-1})	2.0	2.0	2.0	2.0	2.0	0.0
EC_{50} (µg/mL)		4.1	2.8	3.6	3.5	0.7

Table 41: Pharmacodynamic parameters; M. cat

	Initial Cefaclor concentrations (µg/mL)					
Parameters	50	5	0.5; 1-d	0.5; 2-d	Mean	S.D.
k_{max} (h^{-1})	3.0	2.4	2.5	2.5	2.6	0.3
k_0 (h^{-1})	0.8	0.8	0.8	0.8	0.8	0.0
EC_{50} (µg/mL)		1.2	0.2	0.2	0.5	0.6

Table 42: Pharmacodynamic parameters; H. flu

	Initial Cefaclor concentrations (µg/mL)					
Parameters	50	5	20; 1-d	20; 2-d	Mean	SD
k_{max} (h^{-1})	1.8	1.5	1.4	1.4	1.5	0.2
k_0 (h^{-1})	1.0	1.0	1.0	1.0	1.0	0.0
EC_{50} (µg/mL)		3.6	0.9	2.7	2.4	1.4

Table 43: Pharmacodynamic parameters; S. pneumo

	Initial Cefaclor concentrations (µg/mL)					
Parameters	50	20	5; 1-d	5; 2-d	Mean	SD
k_{max} (h^{-1})	2.9	2.4	2.4	2.4	2.5	0.2
k_0 (h^{-1})	1.6	1.6	1.6	1.6	1.6	0.0
EC_{50} (µg/mL)		0.1	0.3	0.4	0.3	0.2

For each bacterial strain, the initial estimates for k_{max} and k_0 were determined using the data for the growth rate in the absence of cefaclor (N_0), and the data for the total kill curves (N_{tk}). Then k_0 was fixed to the value obtained in that initial fit, the data from the different dose regimens were fitted starting with those initial parameters, and the values of k_{max} and EC_{50} were determined in each case.

Curve Fits

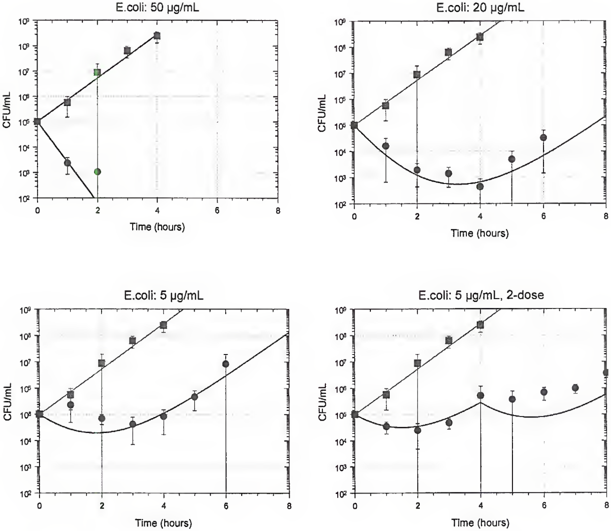


Figure 38: The plots show the number of colony forming units per mL (CFU/mL) (mean ± S.D., n=4) of E. coli for the control experiment (■) and after exposure to the different cefaclor concentrations (●). The lines represent the curve fits from the respective data analysis.

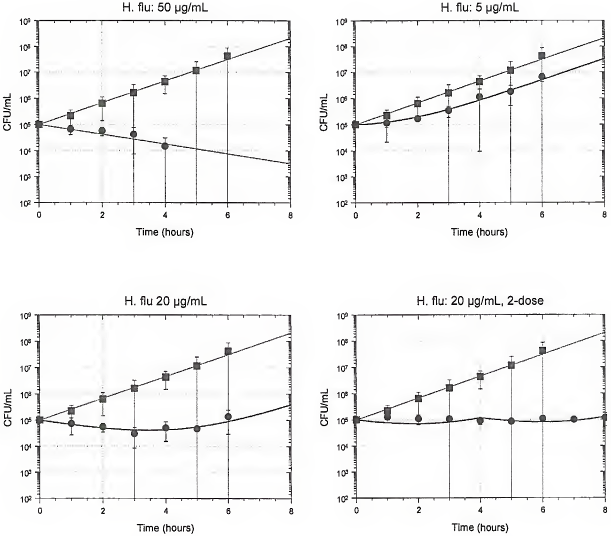
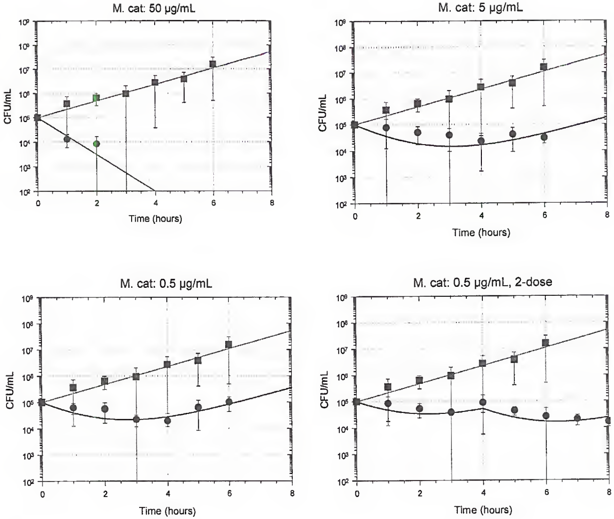


Figure 39: The plots show the number of colony forming units per mL (CFU/mL) (mean ± S.D., n=4) of H. flu for the control experiment (■) and after exposure to the different cefaclor concentrations (●). The lines represent the curve fits from the respective data analysis.



M. cat: 50 µg/mL

M. cat: 5 µg/mL

M. cat: 0.5 µg/mL

M. cat: 0.5 µg/mL, 2-dose

Figure 40: The plots show the number of colony forming units per mL (CFU/mL) (mean ± S.D., n=4) of M. cat for the control experiment (■) and after exposure to the different cefaclor concentrations (●). The lines represent the curve fits from the respective data analysis.

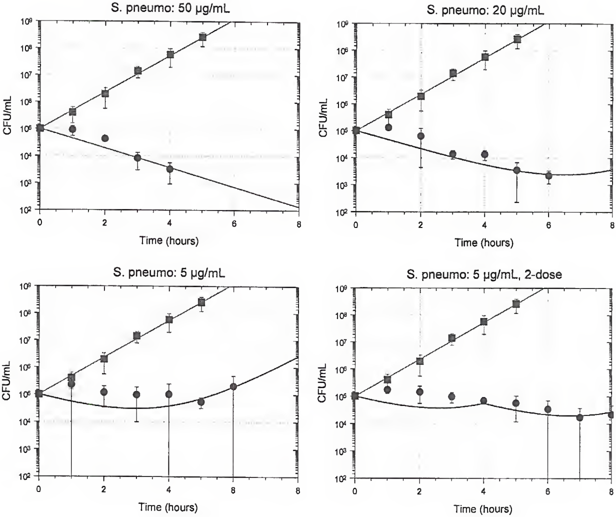
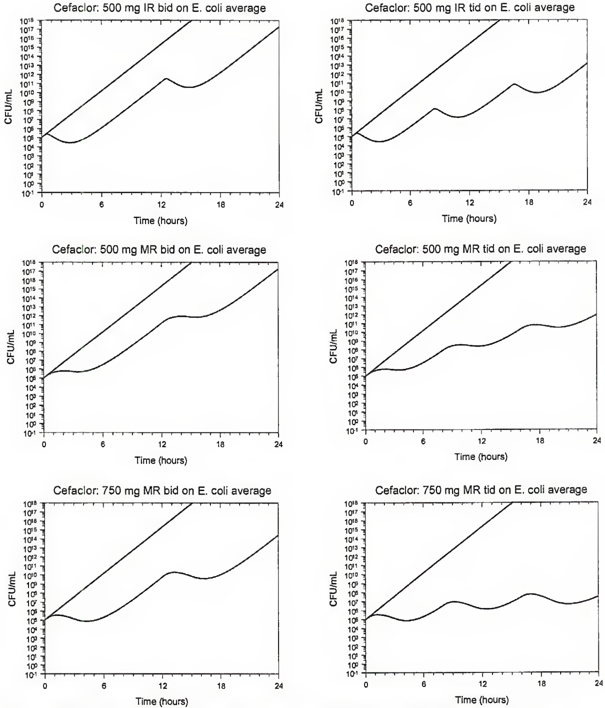


Figure 41: The plots show the number of colony forming units per mL (CFU/mL) (mean ± S.D., n=4) of S. pneumo for the control experiment (■) and after exposure to the different cefaclor concentrations (●). The lines represent the curve fits from the respective data analysis.

Simulations



Cefaclor: 500 mg IR bid on E. coli average

Cefaclor: 500 mg IR tid on E. coli average

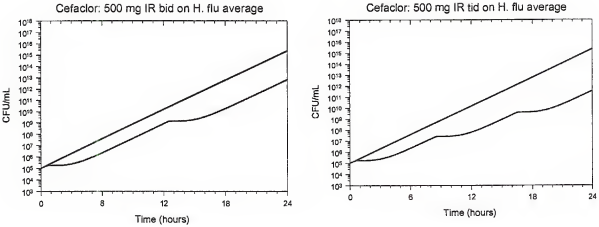
Cefaclor: 500 mg MR bid on E. coli average

Cefaclor: 500 mg MR tid on E. coli average

Cefaclor: 750 mg MR bid on E. coli average

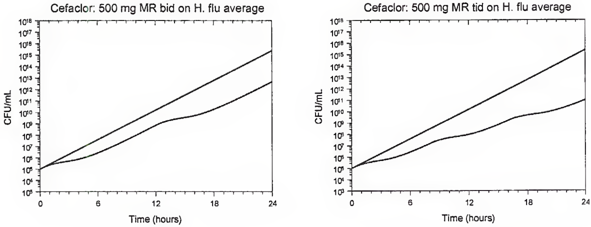
Cefaclor: 750 mg MR tid on E. coli average

Figure 42: PK-PD simulations of the effects of the different cefaclor formulations on E. coli, taking into account mean pharmacokinetic parameters.



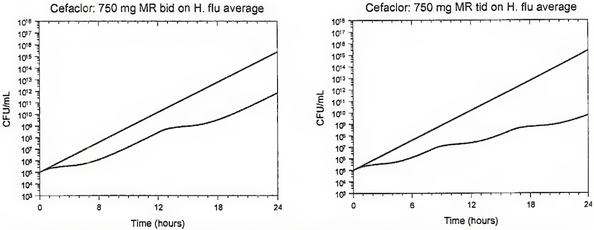
Cefaclor: 500 mg IR bid on H. flu average

Cefaclor: 500 mg IR tid on H. flu average



Cefaclor: 500 mg MR bid on H. flu average

Cefaclor: 500 mg MR tid on H. flu average



Cefaclor: 750 mg MR bid on H. flu average

Cefaclor: 750 mg MR tid on H. flu average

Figure 43: PK-PD simulation of the effects of the different cefaclor formulations on H. flu, taking into account mean pharmacokinetic parameters.

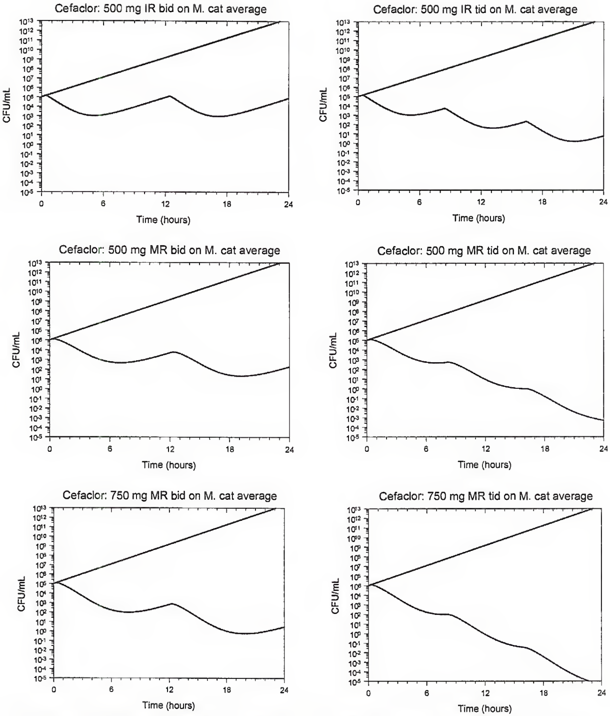
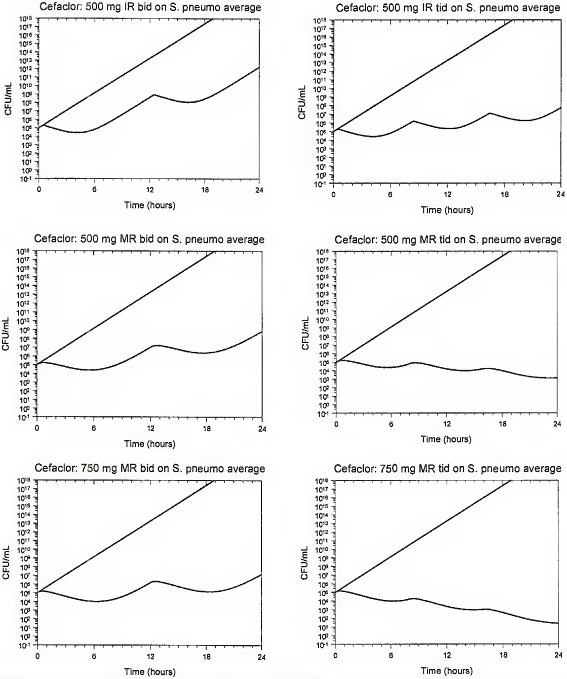


Figure 44: PK-PD simulation of the effects of the different cefaclor formulations on M. cat, taking into account mean pharmacokinetic parameters.



Cefaclor: 500 mg IR bid on S. pneumo average

Cefaclor: 500 mg IR tid on S. pneumo average

Cefaclor: 500 mg MR bid on S. pneumo average

Cefaclor: 500 mg MR tid on S. pneumo average

Cefaclor: 750 mg MR bid on S. pneumo average

Cefaclor: 750 mg MR tid on S. pneumo average

Figure 45: PK-PD simulation of the effects of the different cefaclor formulations on S. pneumo, taking into account mean pharmacokinetic parameters.

Simulation of the Effects of Different Doses and Administration Regimens of Cefaclor on 4 Different Bacterial Strains.

Table 44: CFU/mL remaining after 24 hours (N_{24h})

Dose	E. coli	H. flu	M. cat	S. pneumo
No drug	6.3E+25	2.6E+15	2.1E+13	4.4E+21
500 IR bid	2.4E+17	6.7E+12	8.3E+04	2.1E+12
500 IR tid	1.6E+13	3.8E+11	6.2E+00	5.9E+07
500 MR bid	2.2E+17	4.8E+12	2.0E+02	7.4E+08
500 MR tid	9.7E+11	1.0E+11	5.4E-04	1.4E+03
750 MR bid	3.1E+14	7.3E+11	3.1E+00	1.5E+07
750 MR tid	3.7E+07	5.8E+09	3.8E-06	2.5E+01

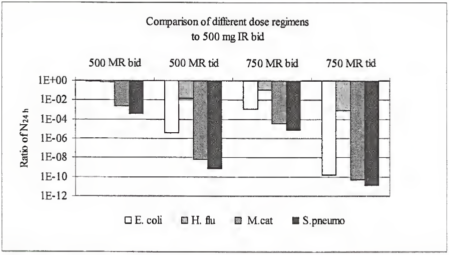


Figure 46: Ratio of N_{24h} of different dosing regimens (500 mg MR bid, 500 mg MR tid, 750 mg MR bid and 750 mg MR tid) to the N_{24h} for a dosing regimen of 500 mg IR bid. From left to right in each bar cluster: E. coli (white), H. flu (light gray), M.cat. (dark gray) and S. pneumo (black)

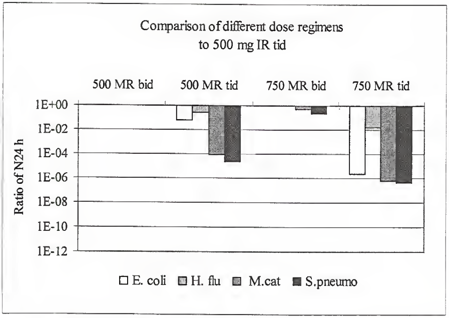


Figure 47: Ratio of N24h of different dosing regimens (500 mg MR bid, 500 mg MR tid, 750 mg MR bid and 750 mg MR tid) to the N24h for a dosing regimen of 500 mg IR tid. From left to right in each bar cluster: E. coli (white), H. flu (light gray), M.cat. (dark gray) and S. pneumo (black)

Discussion

Figure 46 and Figure 47 show the comparison between four different dosing regimens expressed as a ratio of N_{24h} to a 500 mg IR bid (Figure 46) or tid (Figure 47) regimen. N_{24h} is the number of bacteria (CFU/mL) remaining after 24 hours, simulated with the different regimens. A ratio of 1 means equal effect of the two regimens. Any value<1 means the MR dose compared has a bigger effect than the IR dosing regimen.

A 500 mg MR bid regimen shows better results than a 500 mg IR bid regimen only for M. catarrhalis and S. pneumoniae, whereas very little improvement is seen for E. coli and H. influenzae. Changing the regimen to 500 mg MR tid proves to be not only more effective than a 500 mg IR bid regimen, but also than a 500 mg IR tid regimen for all the bacteria studied. The magnitude of the ratio is different for each bacterium studied, being H. influenzae the one that shows the least improvement.

The 750 mg MR bid regimen shows improvement over the 500 mg IR bid regimen for all bacteria, again H. influenzae shows the least improvement. Small advantage is seen for using a 750 mg bid regimen as compared to a 500 mg IR tid, and only for M. catarrhalis and S. pneumoniae. Increasing the dose to a 750 a mg tid regimen proves to be more effective than both the 500 mg IR bid and the 500 mg IR tid regimens for all the bacteria studied. The magnitude of the ratio is different for each bacterium studied, again being H. influenzae the ones that show the smallest improvement.

When designing an appropriate dosage recommendation, the PK and PD parameters need to be taken into account. An important PK parameter that needs consideration is the AUC, which was shown in Chapter 5 to be smaller for the 500 mg MR formulation than for the 500 mg IR formulation. Therefore the effects of the two 500-mg formulations should not be directly compared as if they were pharmacokinetically equivalent. The 750 mg MR formulation, on the other hand, delivers a little more drug than the 500 mg IR formulation, but again, due to the same absorption limitation, the total amount of drug delivered is not proportional to that delivered by the IR formulation. It was considered appropriate then, to compare the 500 mg IR formulation to the 750 mg MR formulation. As shown in Table 44 and Figure 47, for all bacteria strains a 500 mg IR tid dose is equivalent to a 750 mg MR bid dose. The possibility of administering the drug twice instead of three times a day would increase patient compliance. However, from the simulations it can be seen that for E. coli (Figure 42), H. influenzae (Figure 43) and S. pneumoniae (Figure 45) a 750 mg MR bid administration is not enough to produce a reduction in CFU/mL from the initial inoculum after 24 hours. Only in the case of M. catarrhalis (Figure 44) a reduction in N_{24h} is observed. On the other hand, the administration of a 750 mg MR tid regimen still shows some growth compared to the original inoculum in E. coli and H. influenzae, but produces reductions in the N_{24h} for both M. catarrhalis and S. pneumoniae. However, it has to be considered that these results do not include the effect of the immune system of the host, which would be additive to the drug effects. Therefore the recommended dosing regimen for cefaclor would be to administer the 750 mg MR formulation three times a day.

CHAPTER 8
CONCLUSIONS

Although there have been many efforts in the past decade to improve antibiotic dosing regimens, the prevalent tendency nowadays is still empirical rather than a rational PK/PD approach. In an attempt to provide a better dosing method, this study was designed to determine the pharmacokinetics of cefaclor and to investigate the relationship between the tissue concentrations and their anti-infective effect, while evaluating the potential advantages of a new modified release dosage form.

The first step was to determine the drug's stability and to develop an HPLC assay to determine cefaclor concentrations in biological fluids. Protein binding was determined in both human and rat plasma. Previous studies with other beta-lactam antibiotics indicated that rats are a suitable test system to investigate tissue distribution using microdialysis [66, 67, 104]. Animal pharmacokinetic studies were performed in rats and it was determined that the free tissue levels in two different organs (muscle and lung) were identical.

This observation was extrapolated to the human studies, where only free muscle levels were obtained. Unbound tissue concentrations were obtained using microdialysis for both animal and human studies. Predictions of concentration-time profiles of free cefaclor in tissue could be made with reasonable accuracy in both species. The results of the human pharmacokinetic studies show that the two modified release products allow to take advantage of the full absorption window for cefaclor to extend the duration of absorption to approximately three hours [211, 221, 222].

Human tissue pharmacokinetic profiles were then simulated in an *in vitro* model against different bacterial strains in order to determine the pharmacodynamic effect (change in number of bacteria). A PK/PD model that allows for simulation in the number of bacteria vs. time for various dosing regimens of the modified release and the immediate release cefaclor dosage form was developed. Different regimens and formulations were subsequently compared using the ratio of the remaining bacteria after 24 hours, with values of ratio of N_{24h} greater than 1 meaning a greater effect. The PK/PD model was successfully applied to link the cefaclor concentrations to the respective anti-infective activity. This model establishes the quantitative relationship between dosing regimen, pharmacokinetics and antibiotic activity.

The results show that the number of bacteria of all investigated strains killed per day is equivalent when the same daily dose is administered twice a day with the modified release dosage form than when given three times a day with the immediate release dosage form. This is in spite of the fact that the modified release dosage form has approximately 20% lower bioavailability. Best results are obtained with the three-times a day regimen of the modified release formulation.

The pharmacodynamic results were evaluated using both the traditional method based on the MIC and the kill-curve method. From the kill curves approach it was observed that H. influenzae showed the least sensitivity to the drug, as evidenced by the simulations and resulting N_{24h}. This fact did not arise from the traditional approach, where high t>MIC values for H. influenzae were obtained, implying good efficacy of cefaclor. Therefore, by using the t>MIC for the plasma data, an erroneous conclusion would have been reached. According to this data, a 500 mg MR bid dose would have been considered efficacious (%t>MIC approximately 45% for plasma), thus resulting in a poor clinical outcome. An inappropriate antibiotic treatment, in addition to unnecessarily prolonging the disease state and the discomfort to the patient, may lead to the development of resistance, as well as increasing monetary costs.

The inverse situation is observed with S. pneumoniae, where low t>MIC values would lead to the conclusion that cefaclor would not be efficacious against it. Nevertheless, the N_{24h} data derived from the kill curves indicates S. pneumoniae as being the second most sensitive strain to cefaclor, immediately following M. catarrhalis. Again, making a dosing decision based on the t>MIC data would lead to an erroneous choice.

MIC measurements provide a (figuratively speaking) "still-photo" of the effect of an antibiotic at only one fixed concentration value. On the other hand, kill curves are able to show the behavior of the antibiotic due to variable concentration profiles that are a better reflection of what is really happening *in vivo* along time. Another disadvantage of the MIC approach is that it is often regarded as an "all or nothing" threshold value, which would imply the antibiotic activity would be turned "on" or "off" above or below the MIC limit respectively. This is non-realistic because antibiotics do have an effect even at very low concentrations, although it may be a stasis instead of a killing effect.

The discrepancies observed between the methods may be due to the fact that MICs are measured using two-fold dilutions, so that replicate measurements can result in MIC ranges rather than single MIC values. Nevertheless, when t>MIC values are calculated, a single MIC value is utilized. Therefore, the broader the range of MIC values obtained, the greater the error that is made when calculating the t>MIC.

Furthermore, the usual practice is to compare the total plasma levels of a drug to the MIC measured in serum. This does not take into account either that the unbound drug levels are the only available to exert a pharmacological effect or that the unbound levels at the site of action and not those measured in plasma/serum are the relevant ones.

Finally, in the case of cefaclor, its chemical instability should be taken into account when making dosing decisions based on MIC values. MICs are traditionally determined under the assumption that the amount of drug present in each dilution tube is maintained constant throughout the incubation period. As shown in Chapter 3, only a very small percentage (2-14%) of the initial concentration of cefaclor remains in the bacterial growth media after the incubation period. Therefore the MIC determined after this period would not correspond to the initial concentration in the tube, but would need to be corrected according to the precise incubation time and temperature. This fact further supports the use of kill curves versus MICs.

In summary, the kill curve approach provides a better insight into the behavior of the antibiotic against the bacteria along time, as well as giving the possibility of evaluating different dosage regimens, based on clinically relevant concentrations.

When applying the results of these *in vitro* studies to a clinical situation, several factors that can affect the ultimate result need to be taken into account:

- Bacteria may grow differently in vivo and in vitro.
- Small changes in temperature and environmental pH are capable of affecting the stability of cefaclor.

- The contribution of the immune system is not built into the model. Therefore, the *in vivo* effect is probably amplified with respect to in vitro unless the host is immunocompromised.

In spite of these limitations, the developed in vitro model allows comparison of different dosage regimens, to which the immune response will be added as a constant. This method allows to optimize treatment schedules and guide in dosage selection.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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